

Genetic Disruption of Ki-67 Results in Decreased Clonogenic Proliferation

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ABSTRACT

Two well-known hallmarks of cancer are the abilities to sustain proliferation and invade surrounding tissue, and pathways involved in these processes have been scrutinized for possible therapeutic targets in recent years. Since its discovery, Ki-67 has been intimately linked to the cell cycle and widely accepted as a marker for the growing fraction of cells in a tumor sample, and more recently its potential as a prognostic and predictive marker for various cancers has been explored. Despite the wide use of Ki-67 in the clinical and pathological setting, little is known about its actual function and role as a potential therapeutic target for cancer. To better characterize Ki-67's function to optimally exploit this gene/protein for prognostic, predictive, and therapeutic purposes, we created isogenic Ki-67 knockout cells in a “normal” and cancer cell line. Unlike previous efforts to knockdown Ki-67, we were able to generate multiple independent Ki-67 knockout clones in two cell lines that definitively lacked functional full length Ki-67 protein. In colony formation assays *in vitro* and tumorigenic assays *in vivo*, the knockout clones displayed a reduced proliferative and clonogenic potential, illustrating how inhibition of Ki-67 could result in a decreased ability of cancer cells to invade and colonize surrounding and distant tissue. A current model of metastasis maintains that cancer stem cells (CSCs) are required to establish tumor formation at distant sites, and in colorectal cancers, those CSCs are often identified by the expression of cell surface markers CD133 and CD44. The knockout clones had a markedly reduced CD133⁺CD44⁺ population of cells compared to the parental cell line, demonstrating that loss of Ki-67 resulted in fewer colonies and tumors by decreasing the “stemness” of the cells. CSCs are known to be resistant to various chemotherapies, but we found that the loss of Ki-67 in the cancer cell line

resulted in sensitization to multiple chemotherapeutic agents. These findings combine to establish that Ki-67 is a viable therapeutic target, particularly in the metastatic setting. By specifically inhibiting Ki-67, it could be possible to greatly decrease the risk of micrometastatic disease that is often times refractory to standard therapy.

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One of my favorite speeches comes from Michael Lewis, the author of “The Blind Side,” when he gave the commencement address at Princeton University’s 2012 Baccalaureate, and it seems particularly pertinent at this juncture in my life.

“People really don’t like to hear success explained away as luck – especially successful people. As they age, and succeed, people feel their success was somehow inevitable. They don’t want to acknowledge the role played by accident in their lives...Don’t be deceived by life’s outcomes. Life’s outcomes, while not entirely random, have a huge amount of luck baked into them. Above all, recognize that if you have had success, you have also had luck – and with luck comes obligation. You owe a debt, and not just to your gods. You owe a debt to the unlucky.”

I am extraordinarily lucky to have the support, guidance, friendship, and love from so many people, without whom there is no amount of hard work that would have given me the opportunity to earn a Ph.D. from Johns Hopkins School of Medicine. And I’d like to acknowledge those amazing people here.

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TABLE OF CONTENTS

Title Page	i
Abstract	ii
Acknowledgments	iii
Table of Contents	v
List of Figures	vi
List of Tables	vii

Chapters

1. Introduction	
Discovery	1
Gene Structure, Cellular Localization, and Protein Domains	2
Functional Genomic Studies	6
Clinical Utility	7
Hypothesis	9
2. Materials & Methods	11
3. Results	22
4. Discussion	54
References	62
Curriculum Vitae	71

LIST OF FIGURES

Figure 1: Schematic for targeted knockout of <i>MKI67</i>	35
Figure 2: Confirmation of the knockout of Ki-67 in MCF-10A and DLD-1 cell lines	36
Figure 3: Reduced proliferative potential of isogenic cell lines <i>in vitro</i>	38
Figure 4: Reduced proliferative and clonogenic potential of Ki-67 knockout cell lines <i>in vivo</i>	40
Figure 5: Cancer stem cell population decreased in Ki-67 knockout cell lines	42
Figure 6: Knockout of Ki-67 in DLD-1 cells sensitizes them to chemotherapy	43
Figure S1: Confirmation of Ki-67 knockout via IHC of cell tissue blocks	44
Figure S2: RPPA heatmap for the parent and Ki-67 knockout cell lines	45
Figure S3: Conditioned media does not rescue the reduction in growth and/or clonogenic potential of Ki-67 knockout cell lines	46
Figure S4: Loss of Ki-67 does not cause chromosomal instability	47
Figure S5: Diagram of pCMV6-XL4-Ki67 overexpression vector	49
Figure S6: Schematic for knock in of constitutive promoter at the endogenous MKI67 locus	50
Figure S7: Rescue vector restores Ki-67 expression in knockout cell lines	51

LIST OF FIGURES

Table 1: Cloning, screening, and qRT-PCR primers	52
Table 2: Change in Ki-67 expression between matched primary and metastatic breast carcinomas	53

1 Introduction

DISCOVERY

Understanding the basic mechanisms of cell proliferation has led to major insights and advances in physiology and medicine. For cancer research, however, the identification of genes and proteins that are potential targets for therapy is an ongoing quest. Recently there has been interest in targeting Ki-67 for cancer therapy given the fact that aggressive cancers express this protein at high levels while quiescent cells show a complete absence of Ki-67 protein [1–3]. Although there is conflicting data regarding the expression level of Ki-67 in normal dividing cells [4, 5], Ki-67 expression has become the accepted marker for determining the growing fraction of cells in histopathological samples, particularly human neoplasms [6].

Ki-67 was originally identified by Gerdes *et al.* in 1983 as a monoclonal antibody that reacted with nuclei of the Hodgkin's lymphoma cell line, L428 when injected into mice [7]. They then discovered that the antibody reacted similarly against a variety of

other human cancer cells but not against normal peripheral blood lymphocytes and monocytes. Likewise, when the group chemically induced differentiation of HL-60 cells to mature macrophages and stained with Ki-67 at various intervals, expression of Ki-67 was increasingly suppressed. This led them to believe Ki-67 could be a “potent tool for easy and quick evaluation of the proportion of proliferating cells in a tumor.”

GENE STRUCTURE, CELLULAR LOCALIZATION, and PROTEIN DOMAINS

It was still another decade before Ki-67 was characterized as a large nuclear protein through cloning and sequencing of a cDNA library [8, 9]. Schluter *et al.* determined the complete nucleotide and predicted amino acid sequences, identifying 15 exons that correspond to two predominant splice isoforms of the Ki-67 gene (now known as *MKI67*, located on 10q26). These two isoforms correspond to open reading frames of 9,768 and 8,688 base pairs that encode for proteins with calculated molecular weights of 360 and 320 kD, respectively. The difference between the two splice isoforms was the absence of exon 7 from the smaller transcript. But their most intriguing finding was that 6,845 base pairs of the open reading frame all lay within exon 13, which contained 16 repetitive elements, each 122 amino acids in length that share 43 – 62% homology. Within these “Ki-67 Repeats” was a highly conserved stretch of 22 amino acids, known as the “Ki-67 Motif,” that shared 72 – 100% identity and comprised the epitope recognized by the Ki-67 antibody. In the same study, they also identified multiple PEST sites, regions rich in proline, glutamic acid, serine, and threonine that purportedly designate the protein for rapid proteolysis. This aligns with previous findings that Ki-67 is rapidly degraded after mitosis, with a half-life of about 1 – 1.5 hours [10, 11].

Detailed cell cycle analysis revealed that Ki-67 localizes to distinct regions of the nucleus during all active (non-G₀) stages of the cell cycle [12–17]. During interphase and early-G₁ phase, Ki-67 protein is primarily organized around heterochromatic, satellite DNA regions, such as the centromere and telomeres [17]. Then as the nucleoli begin to reassemble during mid-to-late-G₁ phase, Ki-67 can be found localized in those structures. The nucleolus is comprised of three elements: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The processing of ribosomal RNA (rRNA) and associated proteins occur in these areas, progressing from the DFC to the GC [18, 19]. However, Ki-67 does not appear to be involved in the assembly of ribosomes since it does not co-localize with fibrillarin, a highly conserved nucleolar protein associated with small nucleolar RNAs (snoRNAs) and involved in the assembly of ribosomes in the DFC [20]. Therefore, it is accepted that Ki-67 localizes to the “fibrillarin-deficient” region of the DFC. During S phase, Ki-67 expression begins to increase, with protein levels peaking during metaphase and gradually decreasing thereafter [13, 15, 21, 22]. It has been observed that Ki-67 is concentrated around the nucleolar foci during this time while also somewhat present in the nucleoplasm [15, 16, 21, 22]. As the cell transitions into mitosis, Ki-67 disperses from the dissociating nucleoli to the condensed chromosomes and chromosomal periphery [14]. This is also the only time when Ki-67 is reported to be detectable in the cytoplasm [23]. Toward the end of mitosis, Ki-67 is once again localized around nuclear structures, likely the heterochromatic regions, as the cell progresses toward the next G₁ phase.

Studies have shown that these spatial and temporal expression patterns are regulated by cell cycle-specific, post-translational modifications [23–25]. In particular,

these groups demonstrated that Ki-67 is phosphorylated by the CDK1/Cyclin B complex during mitosis. This evidence was confirmed both *in vitro* and *in vivo*, which included the binding of these phosphorylated forms of Ki-67 with the monoclonal MPM-2 antibody. The MPM-2 antibody recognizes a mitosis-specific phosphorylated epitope that is present in a number of different proteins that are active during mitosis [26–28]. There is evidence that many of the phosphorylated MPM-2 antigens are required for the onset of mitosis and that dephosphorylation of those epitopes is necessary for exit of mitosis. So the discovery that Ki-67 is recognized by the MPM-2 antibody at the beginning but not the end of mitosis demonstrates that phosphorylation likely regulates the function of Ki-67 during mitosis [24].

In 1995, Hofman and Bucher identified a forkhead-associated domain (FHA) in twenty seemingly unrelated proteins through advanced sequence profiling methods. One of those proteins was Ki-67 [29]. Upon further inspection, they found that most of those identified proteins also contain a nuclear localization signal and are involved in cell cycle regulation. More recent studies have revealed that these FHA domain-containing regulatory proteins, kinases, phosphatases, and transcription factors play crucial roles in cell growth and signal transduction, particularly in relation to the DNA damage response. Unlike other signal transduction domains that recognize phosphoproteins, the FHA domain is the only one that is phosphothreonine (pThr)-specific whereas most others recognize both pThr and phosphoserine (pSer). Despite the specificity for the pThr residue, the surrounding sequence motifs recognized by the FHA domain are quite diverse and completely dependent upon the particular FHA-containing protein [30, 31]. Through yeast two-hybrid screenings, the human kinesin-like protein 2 (Hklp2) and the

putative RNA-binding human nucleolar interacting with the FHA domain of Ki-67 (hNIFK) were identified as interacting partners with the FHA domain of Ki-67 [32, 33]. Both proteins were also able to interact with GST-FHA(Ki-67) fusions, especially after the proteins were phosphorylated with cyclin/cyclin-dependent kinase complexes. Hklp2 (also known as Kif15) participates in the assembly and stabilization of the bipolar spindle, localizing to the chromosome through Ki-67 [34–36]. In fact, when Ki-67 was silenced with siRNAs, Hklp2 failed to localize to the chromosomes, resulting in much longer bipolar spindles. Combined with the detailed cell cycle analysis and localization studies, it is likely that Ki-67 plays a role in the progression of the cell cycle through the dynamic interactions of its FHA domain with other cell cycle-related proteins.

A third predicted protein domain was identified in 2002 by Scholzen *et al* [37]. They found that the last 220 amino acid residues of the C-terminus of Ki-67 could bind to all three mammalian heterochromatin protein 1 (HP1) isoforms through a series of co-immunoprecipitation and yeast two-hybrid assays. Specifically, through immunofluorescence assays, they found that Ki-67 co-localized with HP1 during interphase of cell cycle. HP1 is a small, non-histone chromosomal protein identified as a component of heterochromatin that acts as a dosage-dependent modifier of position effect variegation [38, 39]. All three isoforms consist of two highly conserved domains: the N-terminal chromodomain (CD), which recognizes methylated histone H3K9 for chromatin binding, and a C-terminal chromoshadow domain (CSD) that is thought to be responsible for protein-protein interactions. This interaction with the gene-poor, repetitive, transcriptionally inert, and late-replicating heterochromatic regions of the genome lend a wide range of functions for the HP1 proteins. Amongst these are transcription silencing

and activation [40–43], telomere capping and silencing [44], DNA replication and repair [45, 46], and mitosis [47, 48]. Although the functional significance of the interaction between Ki-67 and HP1 has not yet been clarified, it is believed that Ki-67 could be responsible for or involved in higher order chromatin structure.

The association of Ki-67 with mitotic chromosomes, HP1 proteins, and the kinesin-like Hklp2 protein, along with its spatial and temporal expression patterns lead many to speculate that Ki-67 is integrally involved in cell growth and division. Consequently, many believe that Ki-67 is an essential gene/protein, vital to the survival of the cell, leading a few groups to use functional genomics approaches to interrogate this hypothesis.

FUNCTIONAL GENOMIC STUDIES

Since Ki-67 is highly expressed in proliferating cells, various groups have tried using some form of RNA interference (RNAi) to silence or knock down the expression of *MKI67* in hopes of determining the effect on cell viability and cellular processes, particularly whether Ki-67 could be a viable therapeutic target in a number of cancers. The first study was conducted by Duchrow *et al.* and used antisense-RNA (asRNA) and recombinant protein to silence *MKI67* expression. They reported an overall slower incorporation of BrdU into cells and progression through the cell cycle [49]. Another group published three separate studies, all utilizing antisense oligonucleotides (asRNA) to knock down *MKI67* expression. They did so in various cancer cell lines as well as *in vivo* models of bladder cancer, prostate cancer, and renal cell carcinoma. They observed decreased *MKI67* transcript and protein expression, inhibited cell growth, and increased

apoptosis in monolayer and 3-D culture after treatment with the asRNA. Similarly, the xenograft tumor volumes were significantly inhibited in the antisense-treated mice [50–52]. And much like the previous group, Zheng *et al.* published four different studies where they knocked down *MKI67* expression using peptide nucleic acids (PNAs), small interfering RNAs (siRNAs), or short hairpin RNAs (shRNAs), noting a marked reduction in cell proliferation and increase in apoptosis [53–56]. For many people, these studies represent further proof that Ki-67 is vital for cell growth and proliferation. But given the caveats that accompany RNAi experiments, such as knockdown efficiency and specificity, it is difficult to make any definitive conclusions.

CLINICAL UTILITY

In the clinical setting, Ki-67 is one of a number of biomarkers frequently used as prognostic indicators for early breast cancers (EBCs) [57, 58]. It can be measured using one of two approaches: a semi-quantitative analysis of *MKI67* mRNA extracted from frozen or formalin-fixed paraffin embedded (FFPE) tissue, or a score based on the percentage of cancer cells that stain positively for Ki-67 protein by immunohistochemistry (IHC). One study reports a strong linear correlation between the two methods when assessing a panel of 53 breast cancers. However, IHC staining has a number of advantages, like the ability of the pathologist to exclude contaminating non-cancer cells. It is also less costly and more accessible in a routine diagnostic laboratory.

As expected, Ki-67 staining strongly correlates with tumor grade since mitotic index is one of the three grading components [59, 60]. Despite evidence from early studies that Ki-67 is a reliable single prognostic indicator, its use as a stand-alone marker

has become controversial [61, 62]. There are some studies that claim Ki-67 can be used as a single biomarker for both relapse prognoses and predictions for therapeutic response. Others have reported that Ki-67 is neither prognostic nor predictive [62], prognostic but not predictive [61][63], or prognostic only as an on- or post-treatment intermediate biomarker [64, 65]. A contributing factor to the disparity amongst these meta-analyses is a lack of established Ki-67 staining, scoring, and reporting protocols. Attempting to harmonize methodologies in order to validate clinical applications of Ki-67 assessment, the International Ki-67 in Breast Cancer Working Group published a number of recommendations for standardized Ki-67 evaluation [66].

Despite the potential shortcomings of Ki-67 as a single prognostic and predictive indicator, its incorporation as one of multiple genes in the Oncotype DX assays has led to its use in both a prognostic and predictive setting for estrogen receptor (ER) positive, node negative, tamoxifen treated, early breast cancers (EBCs) [67–69]. Similar Oncotype DX assays are also available for prostate and colon cancers. Since roughly 60 – 75% of women with EBC are needlessly treated with adjuvant chemotherapy and exposed to the associated risks, better methods are needed to stratify patients into groups of low, intermediate, and high risk of recurrence so that better informed treatment decisions can be made. The Oncotype DX breast cancer assay evaluates the expression level of 21 genes (16 cancer-related genes and 5 reference genes), of which one is *MKI67*, known to be associated with tumor proliferation, invasion, and estrogen signaling [70]. This expression data is combined with clinical-pathological data into an algorithm that determines a 10-year distant recurrence score that can help predict chemotherapy benefit. Therefore, the greater the level of *MKI67* expression along with other genes, the worse

the prognosis and the higher likelihood of recurrence [71]. According to current American Society of Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) guidelines, and until the full results of the TAILORx clinical trial is reported, within the context of Oncotype DX, *MKI67* and other genes can serve as a reliable predictive indicator of response to chemotherapies.

HYPOTHESIS

Despite the utility of Ki-67 as a proliferation marker for clinical-pathology samples and a prognostic and predictive indicator for various cancers, a current unmet need is the lack of clarity regarding its function, which will be necessary for validating Ki-67 as a true target for cancer therapy. The fact that Ki-67 is such a large protein and extremely sensitive to protease cleavage makes it extremely difficult to use in biochemical assays, which likely has led to the uncertainty surrounding its molecular mechanism [9, 72]. Also contributing to the lack of information regarding Ki-67 is the limited capacity of past technologies to completely abrogate its function, highlighted by the few aforementioned functional genomic studies utilizing various forms of RNA interference (RNAi) to knock down *MKI67* expression in various cancer cell lines. While each reports moderate knockdown efficiency that correlates with reduced cell proliferation and increased apoptosis, lending further credence to the belief that Ki-67 is an integral protein in the regulation of cell division and survival [2, 50, 55, 56], none conclusively demonstrate the involvement of Ki-67, either through rescue experiments or the use of multiple RNAi sequences. In this study, we investigate the role of Ki-67 in cell proliferation and tumor initiation by utilizing gene targeting to create mutant Ki-67 knockout human somatic cell

lines that ablate all functional Ki-67 protein. Since it has been reported that some dividing cells did not stain for Ki-67, we expect that knock out of Ki-67 will not affect the viability of the cell lines, but we hope to assess the effects on various other cellular processes.

2 **Materials and Methods**

Cell culture conditions

MCF-10A [73] and its derivative cells were grown in DMEM:F12 medium (Invitrogen) supplemented with 5% horse serum (HS, Hyclone), 20ng/mL epidermal growth factor (EGF, Sigma), 10µg/mL insulin (Sigma), 0.5µg/mL hydrocortisone (Sigma), 0.1µg/mL cholera toxin (Sigma), and 1% penicillin/streptomycin (Invitrogen). This is referred to as “standard growth” media. For “physiologic EGF” conditions, we use standard growth media but replace 20ng/mL EGF with 0.2ng/mL EGF. For EGF-free, “growth arresting” conditions, phenol red-free DMEM:F12 medium and 1% charcoal dextran-treated FBS (CD) were used in place of the standard medium and serum, respectively. This is referred to as “growth arrest” media. Standard growth media for DLD-1 and its derivative cells was McCoy’s 5A medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin. All cells were maintained in a 37°C incubator with 5% CO₂.

Targeted knock out of MKI67

Targeting vectors were designed as previously described [74, 75]. Briefly, homology arms were PCR-amplified from MCF-10A genomic DNA (gDNA) and ligated into the promoterless gene-targeting vector pSEPT. The homology arms and intervening neomycin selection cassette (consisting of splice acceptor, IRES, *neo* ORF, polyadenylation signal, and flanking loxP sites) was then subcloned into an adeno-associated virus (AAV) vector backbone. After packaging in HEK-293T cells, targeting vectors were transduced into MCF-10A or DLD-1 cells, and antibiotic selection was performed using 120µg/mL and 500 µg/mL of G418 (Invitrogen), respectively. Neomycin-resistant colonies were expanded and screened for homologous integration of the targeting vectors via our previously described PCR-based method [76]. Single-cell clones were then infected with an adenovirus encoding Cre recombinase to remove the selection cassette, followed by single-cell dilution and screening by PCR to confirm successful Cre recombination. Cloning and screening primers are listed in **Table 1**.

Quantitative real-time, reverse transcription PCR (qRT-PCR)

Total RNA was prepared from cells using RNeasy Plus kit (Qiagen). cDNA was synthesized with a First-Strand cDNA Synthesis kit (GE Biosciences). PCR amplification was done using the MyIQ system (BioRad) with Platinum Taq polymerase and SYBR green dye (Invitrogen). Please refer to **Table 1** for primer sequences. Human *MKI67* cycle number was normalized to the average of the *ACTB* cycle numbers using the ΔC_t method.

Proliferation and chemotherapy assays

MCF-10A isogenic cells were seeded at least in triplicate at 1×10^3 cells per well in solid black 96-well plates (Corning) in growth arrest media conditions overnight. The media was changed the following day to either standard growth or growth arrest media for the duration of the experiment. DLD-1 isogenic cells were seeded in the same manner, except standard growth media was used throughout. On specified days, cell viability was assessed by CellTiter-Glo according to manufacturer's protocol. For proliferation assays in the presence of chemotherapeutic agents, cells were plated in the same manner. The day after cell seeding, which was considered Day 0, 5-Fluorouracil (5-FU, Sigma), Paclitaxel (Sigma), Cisplatin (Sigma), and Doxorubicin were added to the appropriate assay media and cells. Cell viability was then assayed via CellTiter-Glo on Day 6 of the experiment.

Clonogenic assays

The isogenic MCF-10A and DLD-1 cells were plated at either 2.5×10^2 or 1×10^3 cells per well in 6-, 12-, 24-, and 48-well plates in standard growth media. The cells were allowed to form colonies for 10 days before being fixed and stained with 0.2% crystal violet (w:v) in 10% buffered formalin. All colonies visible by eye were counted.

Single cell clonogenic assays for MCF-10A and DLD-1 cells were plated in five 96-well plates per clone at a density of 0.5 and 1.0 cells/well, respectively, in 175 μ L of standard growth media. Clonogenicity is scored by counting the number of wells with

colonies per plate per clone, and relative colony size was also documented. MCF-10A cells were scored after two weeks and DLD-1 cells were scored after three to four weeks.

For colony formation assays in semisolid medium, 1×10^2 or 1×10^3 exponentially growing cells were cast in 400 μ L of top layer medium composed of 2% GelTrex LDEV-Free Reduced Growth Factor Basement Membrane (Gibco) in standard growth medium and poured on top of 100 μ L pure GelTrex LDEV-Free Reduced Growth Factor Basement Membrane in 8-chamber tissue culture treated glass slide (BD Falcon). Media plus basement membrane mixture was replaced every four days until the colonies were visualized and photographed after ten days of incubation.

Cell line immunohistochemical and western blot analysis

Cell line paraffin-embedded blocks were prepared from exponentially growing or fully arrested cells in T75cm² flasks. MCF-10A cells were cultured in either standard (20ng/mL EGF, 5% HS) or growth arrest (0ng/mL EGF, 1% CD serum) conditions. Two flasks worth of cells were cultured for each clone. The cells from both flasks were combined following dissociation with trypsin, spun down for 5 minutes at 600 x g, washed twice with HBSS, and centrifuged again. The pellets were resuspended and fixed for 18 hours at benchtop in 10mL 10% buffered formalin, washed twice in PBS, and resuspended in cold PBS (100 μ L total). An agarose plug was prepared for each clone by mixing 100 μ L 2% agarose (w:v) with the resuspended cell pellet, after which the plugs were arrayed in a cassette, paraffin embedded, and processed for 4 μ m-thick slides. For immunohistochemical labeling, the slides were deparaffinized in xylene for 30 minutes and rehydrated using graded ethanol concentrations. Following antigen retrieval, labeling

of Ki-67 (7B11, Invitrogen) was performed using the avidin-biotin peroxidase complex technique and 3', 3'-diamino-benzidine (DAB) as chromogen (Ventana/Biotek Solutions).

Whole-cell protein lysates were prepared from exponentially growing cells in T75cm² flasks. DLD-1 cells were propagated in standard growth conditions while MCF-10A cells were grown under 20ng/mL EGF and 1% CD serum. Cells were dissociated with trypsin for 10 minutes (0.25% Trypsin-EDTA, Invitrogen), resuspended in standard growth media, and spun down for 5 minutes at 600 x g. The cell pellets were resuspended in 150μL PBS (Invitrogen) supplemented with protease inhibitor (Roche Complete Mini). Then 150μL 2X Laemmli sample buffer (Sigma) supplemented with protease inhibitor was added, mixed thoroughly, and boiled for 10 minutes at 100°C. Relative protein concentrations were determined using the 660nm Protein Assay Reagent with Ionic Detergent Compatibility Reagent (Pierce) to allow equal loading of lysates. Loading amounts were normalized to the least concentrated sample. Protein lysates were resolved by SDS-PAGE on Novex 4% Tris-Glycine gels (Invitrogen) for 95 minutes at 125V, transferred to Invitrolon nitrocellulose membranes (Invitrogen) for 16 hours on ice at 75mA, and probed with primary and horseradish peroxidase-conjugated secondary antibodies. Membranes were blocked and probed in 4% cold water fish skin gelatin (Sigma). The primary antibodies used in this study are anti-Ki-67 (MIB-1, Santa Cruz) and anti-GAPDH (6C5, Abcam).

Transfection of overexpression and fusion constructs

TrueClone *MKI67* (transcript variant 1) and NIFK-GFP expression vectors were purchased (OriGene). For transient transfection, exponentially growing MCF-10A, DLD-1, and the derivative knockout cell lines were transfected with 1 μ g DNA using Eugene6 Transfection Reagent (Promega) in antibiotic-free growth media. The next day either whole-cell protein lysates were prepared as described, scaled for 6-well plates, or pictures were taken using fluorescent microscopy. For stable overexpression, the *MKI67* expression vector was transfected in a 3:1 ratio with pcDNA3.1(-) also using Eugene6 Transfection Reagent, and selection with G418 (Invitrogen) was initiated two days post-transfection.

Reverse phase protein array (RPPA)

Parental MCF-10A and the two isogenic knockout clones were grown for three days in 0.2ng/mL EGF (1% CD serum) media prior to plating. In 6-well plates, the cells were then seeded at either 1x10⁵ cells per well and grown for two days in 20ng/mL EGF media or 7.5x10⁵ cells per well and arrested for two days in 0ng/mL EGF media. Parental DLD-1 and the two isogenic knockout clones were seeded at 2.5x10⁵ cells per well and grown for two days in standard growth medium. After two days, the 75-85% confluent cells were harvested for RPPA lysates using the Lysis Buffer and 4X SDS Sample Buffer supplied by the RPPA Core Facility in MD Anderson Cancer Center. Cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon

BioSystems). Total 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7 were used in reverse phase protein array study. Antibodies with a single or dominant band on western blotting were further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands/inhibitors or siRNA for phospho- or structural proteins, respectively. The signal obtained was amplified using a Dako Cytomation-catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customerized-software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model (“Supercurve Fitting” developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, “<http://bioinformatics.mdanderson.org/OOMPA>”). This fits a single curve using all the samples (i.e., dilution series) on a slide with the signal intensity as the response variable and the dilution steps are independent variable. The fitted curve is plotted with the signal intensities – both observed and fitted - on the y-axis and the log₂-concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

Mouse xenografts

Parental DLD-1 cells and two isogenic knockout clones were propagated for three days prior to serial dilution and inoculation. For each group, ten 8- to 10-week-old female athymic nude mice (Taconic) were injected subcutaneously in the right flank with 200 μ L of 4:1 reduced growth factor Geltrex:HBSS (Invitrogen) mixture containing serial dilutions of the parental and derivative DLD-1 cell lines. Tumor volumes were measured weekly and calculated by multiplying the length, width, and height of each tumor until reaching the upper limit of 1.5cm³. The NIH Guide for the Care and Use of Laboratory Animals was followed in all experiments.

Fluorescent in situ hybridization (FISH)

Exponentially growing cells were dissociated with trypsin and seeded at 3x10⁴ cells per well of an 8-chamber culture slide (BD Falcon) in standard growth conditions. After 48 hours, slides were pretreated using Paraffin Pretreatment Reagent Kits (Abbott) and dehydrated with graded ethanol concentrations. Vysis LSI 22 (BCR) SpectrumGreen and LSI EGFR SpectrumRed probes were applied to the slide, denatured at 95°C, and hybridized overnight at 37°C in a humidity chamber. Slides were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) before allele counting in approximately 200 cells per probe per clone under fluorescence microscopy.

Flow cytometry

DLD-1 parental and HomoKO clones were subjected to flow cytometry analysis for the expression of CD44 and CD133. Briefly, 2 million cells were harvested from 85%

confluent flasks and resuspended in PBS with 0.1% BSA. The cells were washed and incubated at 4°C for 15 minutes with anti-CD44-allophycocyanin (APC) (1:20 dilution, clone G44-26, BD Biosciences) and anti-CD133-phycoerythrin (PE) (1:20 dilution, clone AC133, Miltenyi Biotec) antibodies, or mouse-specific IgG_{2b} κ-APC (1:100 dilution, BD Biosciences) and IgG₁-PE (1:20 dilution, Miltenyi Biotec) antibodies. The cells were then washed and resuspended in PBS with 0.1% BSA and 2µg/mL propidium iodide (PI), and a FACSCalibur flow cytometer (BD Biosciences) was used for all analyses. The cells were first gated on the basis of side-scatter and forward-scatter, followed by the exclusion of nonviable (PI-positive) cells. The CD44⁺ and CD133⁺ gates were created on the basis of cellular staining with the isotype control antibodies (IgG_{2b} κ-APC and IgG₁-PE, respectively).

Overexpression of Ki-67 with constitutive GAPDH promoter at endogenous MKI67 locus

Vectors and targeted clones were generated as described in the “Targeted knock out of MKI67” section, except a minimal CMV or human GAPDH promoter was subcloned into the pSEPT vector downstream of the selection cassette but upstream of the 3’ loxP site. Cloning and screening primers are listed in **Table 1**.

Targeted restoration of wild type MKI67 expression by rescue vector

The vector was designed just like the DOWN knockout vector, as described in the “Targeted knock out of MKI67” section. However, the 5’ homology arm was extended to restore the deleted portion of exon 2 and the adjacent couple hundred base pairs of intronic sequence. Cloning and screening primers are listed in **Table 1**.

Ki-67 staining of matched primary and metastatic breast carcinomas

Ki67 proliferation index was assessed by immunohistochemistry (rabbit monoclonal antibody, clone 30-9, catalog number 790-4286, Ventana Medical Systems, Inc.,) on tissue microarrays (TMAs) containing 19 pairs of matched primary human invasive mammary carcinomas and their subsequent visceral metastases harvested at the time of surgery. TMAs contained 5-10 cores per tumor to minimize sampling error. The TMAs were labeled by immunohistochemistry for Ki67, and the average proliferation index across all cores was calculated for each case in increments of 5% nuclear labeling, from 0% to 100%. Labeling of benign breast lobules and lymphocytes served as positive internal controls. Ki67 proliferation index was considered to be equivalent in the primary and metastasis if the values were the same or within a 5% increment of each other.

If available, the Ki67 proliferation index for the primary or metastatic tumor as reported in the surgical pathology report was also subsequently recorded and compared to the result on the TMA to ensure concordance. One case showed marked discordance in the Ki67 proliferation index of the primary tumor from the surgical pathology report (Ki67 proliferation index of 25-30%) to the TMA value (Ki67 proliferation index of 2%), and this case was discarded from evaluation.

In four cases, the primary tumor Ki67 was unevaluable on the TMA due to processing artifact; in these 4 cases, the reported Ki67 proliferation index from the surgical pathology report was used for analysis. The remaining 14 cases had concordant Ki67 proliferation indices in the primary tumor between the TMA result and the surgical

pathology report. Ki67 was rarely performed on the metastatic tumor in the surgical pathology report (n=4), but those 4 cases did have concordant values with the TMA.

3

Results

Generation of somatic cell Ki-67 knockouts

We generated isogenic somatic cell knockouts of Ki-67 in both MCF-10A and DLD-1 cell lines. MCF-10A cells are a non-tumorigenic, spontaneously immortalized, genetically stable breast epithelial cell line, whereas DLD-1 cells are a microsatellite instable (MIN) colorectal cancer cell line. We used these cell lines primarily because they are both diploid for the *MKI67* locus and susceptible to gene targeting. An added benefit of using MCF-10A cells is that, since they require various exogenous growth factor supplementations for growth, the removal of epidermal growth factor (EGF) causes the cells to fully arrest.

The targeting vectors were constructed to insert premature nonsense (STOP) mutations into the first coding exon (exon 2) of the *MKI67* gene, which would disrupt each open reading frame. Targeted integration of the 11-base pair STOP sequence is achieved via homologous recombination of the transduced targeting vector with the *MKI67* gene locus. By subsequently introducing Cre recombinase transiently, all exogenous bacterial sequences originating from the targeting vector were removed except for the STOP sequence and a 117-base pair sequence surrounding a loxP site in intron 2. For both MCF-10A and DLD-1 cell lines, we established two independently derived Ki-67 knockout clones.

Since these cell lines are diploid for the *MKI67* locus, two successive rounds of gene targeting were required to generate a full knockout. The transduction of the parental

cell line with a targeting vector will yield a heterozygous knockout (HetKO), and the subsequent transduction of the HetKO should yield a homozygous knockout (HomoKO). However, as we have observed across numerous gene-targeting projects in our lab, the use of only one targeting vector will predominantly result in re-targeting of the first targeted allele in the HetKO, making it quite difficult to isolate a HomoKO clone. We therefore designed a system utilizing two targeting vectors that would eliminate the possibility of re-targeting upon the second transduction. To control for mutation location, the two vectors shared the same 5' homology arm and adjoining STOP sequence but contained different 3' homology arms. The 3' homology arm of the "UP" vector targeted a region of intron 2 that resulted in the deletion of 13-base pairs of genomic sequence upon successful recombination. The "DOWN" vector 3' homology arm, on the other hand, targeted a region containing exons 2 and 3 that resulted in the deletion of 1,416-base pairs of genomic sequence, including the genomic region corresponding to the "UP" vector 3' homology arm (**Figure 1A-B**). Therefore, utilizing the "UP" vector for the second round of gene targeting resulted in homologous recombination only with the remaining wild type allele, eliminating excess screening of re-targeted clones. Correct targeting was demonstrated by PCR amplification of gDNA derived from Ki-67 knockout clones after Cre-loxP recombination (**Figure 2**).

When generating HetKO clones from parental MCF-10A or DLD-1 cell lines, we calculated a targeting frequency of roughly 4%, regardless of which vector was used. For the subsequent transduction of the appropriate HetKO clone in generating a HomoKO clone, we would expect to see a targeting frequency of about 2% since only one wild type allele remains. In actuality, we observe a 1.5% targeting frequency. The difference

between the observed and theoretical values could be due to the inability to distinguish between primary targeted cells from daughter cells of those original clones during the selection and screening process. Regardless, targeting frequencies even 10- to 100-fold less would still indicate a real, non-artifactual event. In the instance of attempting to knockout a gene essential to the viability of the cell, you could expect to see rare re-targeting events, of which we detected none, and a 0% targeting efficiency. Therefore, these independently generated HomoKO clones are true isogenic Ki-67 knockout cell lines.

We first tried to verify the knockout of Ki-67 through quantitative real-time RT-PCR (qRT-PCR) mRNA expression analysis. The premature nonsense mutations we incorporated into the first coding exon should result in elimination of those mutant mRNA molecules through the nonsense-mediated decay (NMD) pathway [77]. Therefore, we would expect to see a “full” amount of *MKI67* mRNA in the parental cell lines, about half of the “full” transcript levels in the HetKO clones, and almost no transcript in the HomoKO clones. Interestingly, the resulting levels of detectable *MKI67* transcript by qRT-PCR varied depending upon growth conditions. For instance, when the isogenic cell lines were growth arrested (MCF-10A) or serum starved (DLD-1) for two days and then grown under standard conditions for one day, we observed our expected results. However, if the cells were continually grown under standard conditions for multiple passages before harvesting for qRT-PCR analysis, the *MKI67* transcript levels reached a steady-state amount that was equivalent for all clones (**data not shown**). Although the various growth conditions confounded our results, the observed NMD in

growth arrested and serum starved conditions led us to believe that we had properly knocked-out Ki-67.

MCF-10A cells and the derivative knockouts, propagated in either standard growth (20ng/mL EGF, 5% HS) or growth arrest (0ng/mL EGF, 1% CD serum) media conditions, were processed to resemble tissue blocks by paraffin-embedding agarose plugs of the cells, which were subsequently labeled with a Ki-67 antibody via IHC. Under standard growth conditions, Ki-67 protein levels in the heterozygous knockout (HetKO) clones were unchanged relative to the parental cell line, whereas the homozygous knockout clone (HomoKO) showed a clear lack of Ki-67 protein with only trace amounts of residual, non-specific staining. Since Ki-67 protein is not present in quiescent cells and has such a short half-life, the trace staining evident in the clones propagated in growth arrest conditions indicates that the samples contain artifactual staining (**Supplement 1**). Western blot analysis confirmed the IHC results, establishing that Ki-67 protein levels remain largely unchanged in the HetKOs relative to the parental cell line whereas protein expression is completely ablated in the HomoKOs, which could be restored by transient transfection of the HomoKO clones with a Ki-67 (transcript variant 1) over-expression vector (**Figure 2**).

Loss of Ki-67 does not significantly change protein expression patterns or increase chromosomal instability

Although the known interactions of Ki-67 are limited, we wanted to ascertain a sense of the global effects from the knock out of Ki-67, particularly on downstream effectors. We performed reverse phase protein assays (RPPA) on the isogenic MCF-10A and DLD-1

clones to identify any major changes in protein expression between the parent and HomoKO clones (**Supplement 2**). While some genes did show significantly altered expression, it was not consistent across both MCF-10A and DLD-1 cell lines, leading us to believe that the differences are likely due to clonal variability. Similarly, if Ki-67 is indeed responsible for cell proliferation as many hypothesize, we would expect to see the MCF-10A HomoKO clones grown in Full EGF conditions to have a similar gene expression signature as the parental cell line growth arrested in No EGF conditions. However, this trend is not observed. The only significant differences detected were between growth conditions and cell type. Therefore, we conclude that the loss of Ki-67 does not significantly affect the expression of many of the well-known effectors from various signaling pathways.

We also eliminated the possibility that the loss of Ki-67 affects paracrine signaling after finding that growing the HomoKO clones in conditioned media from the respective parental cell line did not rescue the growth phenotype (**Supplement 3**). However, the conditioned media did cause the HomoKO colonies to morphologically resemble the parental cells grown under normal media conditions even though when grown in monolayers, the parental and HomoKO cells displayed no morphological differences. Also, since other studies have linked Ki-67 to higher-order chromatin structure [14, 32, 37] and DNA damage response (outside discussions), we wanted to determine whether the loss of Ki-67 could cause chromosome instability. Using FISH analysis, however, we did not observe any chromosomal abnormalities (**Supplement 4**).

Effect of Ki-67 knock out on proliferative and clonogenic potential in vitro

It has been reported that knock down of *MKI67* results in decreased cell proliferation and increased apoptosis [50, 55, 56]. We performed 7-day growth assays comparing the proliferative potential of the parental, HetKO, and HomoKO clones of MCF-10A and DLD-1. For the MCF-10A clones, there was no significant difference in growth between any of the clones, but we did observe a marginal significant difference for the DLD-1 parental cell line compared to the HetKO and HomoKO clones ($p < 0.05$, **Figure 3A-C**). Therefore, knockout of Ki-67 displayed no obvious growth inhibitory effects on either MCF-10A or DLD-1 cells.

Since the aforementioned growth assays were performed in 96-well plates, we wanted to assess whether seeding density affected the observed growth rates. Using the same number of cells (1×10^3), we conducted the assays in plates with increasingly larger wells, thereby incrementally decreasing the seeding density. To demonstrate the growth phenotype of the cells under normal propagation conditions, we also seeded 6-well plates with 1:10 dilutions of 70% confluent plates. Interestingly for both the MCF-10A and DLD-1 cells, there was a significant reduction in growth potential of the independent HomoKO clones compared to the respective parental cell line as the seeding density decreased, although the growth rate appears equivalent under typical cell passage conditions as the 96-well growth experiment indicated (**Figure 3D**). This suggests that the absence of Ki-67 causes either reduced proliferative and/or clonogenic potential under sparsely seeded conditions. To clarify which process is being affected by the loss of Ki-67, we seeded five 96-well plates per cell line with a calculated density of 0.5 cells per well, allowing us to count single cell colony growth per plate. For both the MCF-10A

and DLD-1 cells, the HomoKO clones contained the same number of colonies as the parental cell line but were noticeably smaller in colony size. In fact, the parental cell lines contained countable colonies about two weeks before the HomoKO cells formed colonies large enough to analyze (**data not shown**).

Effect of Ki-67 knockout on clonogenic potential in vivo

While the knockout of Ki-67 significantly reduces the proliferative and/or clonogenic potential of MCF-10A and DLD-1 cells when assessed by *in vitro* colony formation assays, we wanted to determine if the effects were observed *in vivo* as well. Since MCF-10A cells are a non-tumorigenic breast epithelial cell line, only the DLD-1 clones could be used for *in vivo* experiments. Four 10-fold serial dilutions ($10^6 - 10^3$ cells) of each DLD-1 clone were injected subcutaneously into athymic nude mice, and the subsequent tumors were allowed to grow until reaching a maximum volume of 1.5cm^3 . Similar to the *in vitro* clonogenic assays, the overall growth rate of the HomoKO tumors was significantly less than that of the parental tumors, as shown by comparing the tumor volumes of the three clones of the same dilution (**Figure 4A**). Moreover, when comparing tumor volumes of the four serial dilutions of the same clone, the reduced clonogenic potential of the HomoKO clones is noticeable, demonstrated by the decrease in tumor formation for all dilutions other than the 10^6 group compared to the parental cell line that showed even dose-response curves (**Figure 4B**).

Stem cell markers and chemotherapy sensitivity

The cancer stem cell (CSC) hypothesis postulates that there is a hierarchical organization of cells within a tumor, an intrinsic heterogeneity, such that a specific subpopulation is responsible for tumorigenesis and metastasis [78, 79]. CSCs have been defined and isolated by a series of cell surface markers whose expression pattern is unique to each tumor type. For colorectal CSCs, identification remains controversial due to the lack of consensus for molecular markers even though multiple have been proposed. However, the markers most commonly used to identify and isolate colorectal CSCs are CD133 and CD44. According to one group, subcutaneous injections of CD133⁺CD44⁺ cells always form xenografts [80], and high levels of double-positive cells were a strong indicator for worse disease-free survival and increased risk of recurrence when identified in primary tumors [81]. In another study, CD133⁺CD44⁺ cells were present in each liver metastasis while the primary tumors contained cells with variable expression levels [82]. Via flow cytometry, we analyzed the isogenic DLD-1 HomoKO cell lines for their pattern of CD133 and CD44 expression. We found 0.86% of the parental DLD-1 cells to be CD133⁺CD44⁺, yet the percentage of double-positive HomoKO cell lines was reduced to 0.20% and 0.165%, respectively (**Figure 5**). This could indicate that the loss of Ki-67 negatively affects the number or quality of CSCs in a population.

Various studies have shown CSCs to be resistant to numerous anti-neoplastic agents, implicating that small population of cells in tumor relapse after therapy [83, 84]. Therefore, much clinical interest lay in the potential to target CSC. In the same vein, as a single gene predictor for response to chemotherapies, Ki-67 expression remains controversial albeit promising; but in the multi-gene setting of the Oncotype DX breast

and colon cancer assays, it has proven to be both prognostic and predictive [57, 62, 71, 85]. Since the knock out of Ki-67 in DLD-1 cells resulted in a reduction of CD133⁺CD44⁺ subpopulation of CSCs, we wanted to assess whether the HomoKO cells displayed altered sensitivities to various classes of chemotherapies. Paclitaxel (microtubule stabilizer), cisplatin (alkylating agent), doxorubicin (DNA intercalator), and 5-fluorouracil (anti-metabolite) were all used at doses that inhibited the growth of the MCF-10A and DLD-1 parental cell lines by about 50% (IC₅₀) over seven days, which would serve as a control for chemotherapeutic activity. Compared to parental MCF-10A cells, the HomoKO clones did not display any significant differences in growth following exposure to each of the aforementioned chemotherapies. On the other hand, the DLD-1 HomoKOs showed a significant increase in sensitivity to each of the chemotherapies compared to the parental cells (**Figure 6**). This decreased resistance to chemotherapies correlates with the decreased number or quality of CSCs in the population.

Failed overexpression of Ki-67 transgene

Since we were able to successfully use a Ki-67 overexpression vector, pCMV6-XL4-Ki67 purchased from OriGene (**Supplement 5**), to transiently transfect MCF-10A and DLD-1 HomoKO clones and restore wild type Ki-67 protein, we wanted to stably overexpress Ki-67 and determine if we can rescue the reduced proliferative and/or clonogenic potential. Since the overexpression vector did not contain a selection marker, we performed a co-transfection with the pcDNA3.1(-) construct, which would confer resistance to neomycin. Using standard transfection protocols for the Fugene 6 transfection reagent, the total quantity of pCMV6-XL4-Ki67 transfected into MCF-10A

and DLD-1 HomoKO cells was anywhere from 3x – 5x the amount of pcDNA3.1(-), thus ensuring any resistant cells would also likely contain the overexpression vector as well based upon Poisson distribution. However, after weeks of selection, we could not detect Ki-67 overexpression in any of the stably resistant clones that resulted.

Knock in of constitutive promoter at endogenous MKI67 locus to overcome growth arrest

To determine whether Ki-67 plays a role in driving cell proliferation, we attempted to knock in a constitutive promoter at the endogenous *MKI67* locus that would express *MKI67* even under conditions of growth arrest when Ki-67 protein is typically absent. The targeting vectors were designed much like the knockout constructs in that two homology arms, which direct the vector to the genomic region of interest, flank loxP sites that sit on either end of a neomycin selection cassette. However, between the selection cassette and downstream loxP site, we cloned in either a minimal CMV or human GAPDH promoter. The vector was designed to target the 5'UTR region of the *MKI67* gene so that the selection cassette was driven from the endogenous promoter, yet the constitutive CMV or GAPDH promoter would drive expression of the *MKI67* coding region (**Supplement 6**).

Based upon mRNA expression analysis through qRT-PCR, we found that MCF-10A cells containing the GAPDH knock in promoter and propagated under growth arresting conditions (0ng/mL EGF) express equivalent levels of mRNA as parental cells grown under physiologic EGF conditions (0.2ng/mL EGF). However, when clonogenic assays were performed using the parental and knockin cells under growth arrest conditions, the knockin cells did not overcome growth arrest to form colonies (**data not**

shown). Therefore, based on these results, we can conclude that Ki-67 does not drive cell proliferation by itself. One caveat to these experiments is that we were only able to generate one knockin clone, so we were not able to reproduce the result in independently derived clones.

Rescue of Ki-67 expression in knockout cells by re-targeting mutant allele with vector that restores coding genomic sequences

We designed another gene targeting vector with only a slight modification compared to the original Ki-67 knockout constructs. This rescue vector was created to re-target one of the already targeted and knocked-out *MKI67* alleles, replacing the STOP sequences with the original exonic sequence that had been deleted and restoring the full length expression of Ki-67. The same homology arms as the “DOWN” knockout vector were utilized, except the 5’ homology arm now excludes the STOP mutations and includes all of exon 2 and a couple hundred base pairs of the downstream intronic sequence, thus ensuring restoration of all necessary coding and splicing sequences (**Supplement 7A**). We were able to successfully homologously integrate the rescue vector into the “UP” vector-targeted allele and restore a wild type Ki-67 expression, which we confirmed both by PCR and western blot for multiple isolated clones (**Supplement 7B**). However, when we performed clonogenic assays comparing the rescued clones to the parental and HomoKO cell lines, we were met with ambiguous results. Some of the clones showed a moderately restored proliferative and clonogenic phenotype while others behaved similar to the HomoKO cells (**data not shown**).

Transiently transfected NIFK-GFP does not display aberrant cellular localization.

Through its FHA domain, it has been shown that Ki-67 interacts with at least two proteins: Hklp2 and NIFK. Using a purchased NIFK-GFP fusion construct (OriGene), we transfected MCF-10A and DLD-1 parental and HomoKO cell lines and observed localization patterns. Although we expected to see aberrant localization of NIFK in the Ki-67 knockout clones, there were no obvious differences between the parental and HomoKO cell lines (**data not shown**).

Change in Ki-67 expression between matched primary and metastatic breast carcinomas

The primary breast carcinomas (n=18) consisted of 17 invasive ductal carcinomas and 1 invasive lobular carcinoma; 5 Elston grade 2 and 13 Elston grade 3; and 11 luminal A phenotype (ER+/PR+/Her2-), 1 Her2 phenotype (ER-/PR-/Her2+) and 6 triple negative (ER-/PR-/Her2-). The metastatic sites (one per patient) consisted of 8 brain, 5 lung, 2 liver, 2 gastrointestinal tract, and 1 paraspinal soft tissue.

The Ki67 proliferation indices were the same in the primary and metastasis in 77% (14/18) cases. The Ki67 proliferation indices were increased in the metastatic site in 22% (4/18) of matched cases. All cases showed an increase in the Ki67 proliferation index by an increment of greater than 20% (**Table 2**). The cases with increased Ki67 in the metastasis were all invasive ductal carcinomas; 2 metastasized to the lung and 2 to the brain; 1 was Her2 phenotype, 2 triple negative phenotype, and 1 luminal phenotype. In total, an increase in Ki67 in the metastasis was seen in 100% (1/1) Her2 cases, 33% (2/6) triple negative cases, and 9% (1/11) luminal cases; and in 40% (2/5) lung metastases, 25%

(2/8 brain metastases, and none of the remaining metastatic sites (liver, gastrointestinal tract or spine soft tissue)).

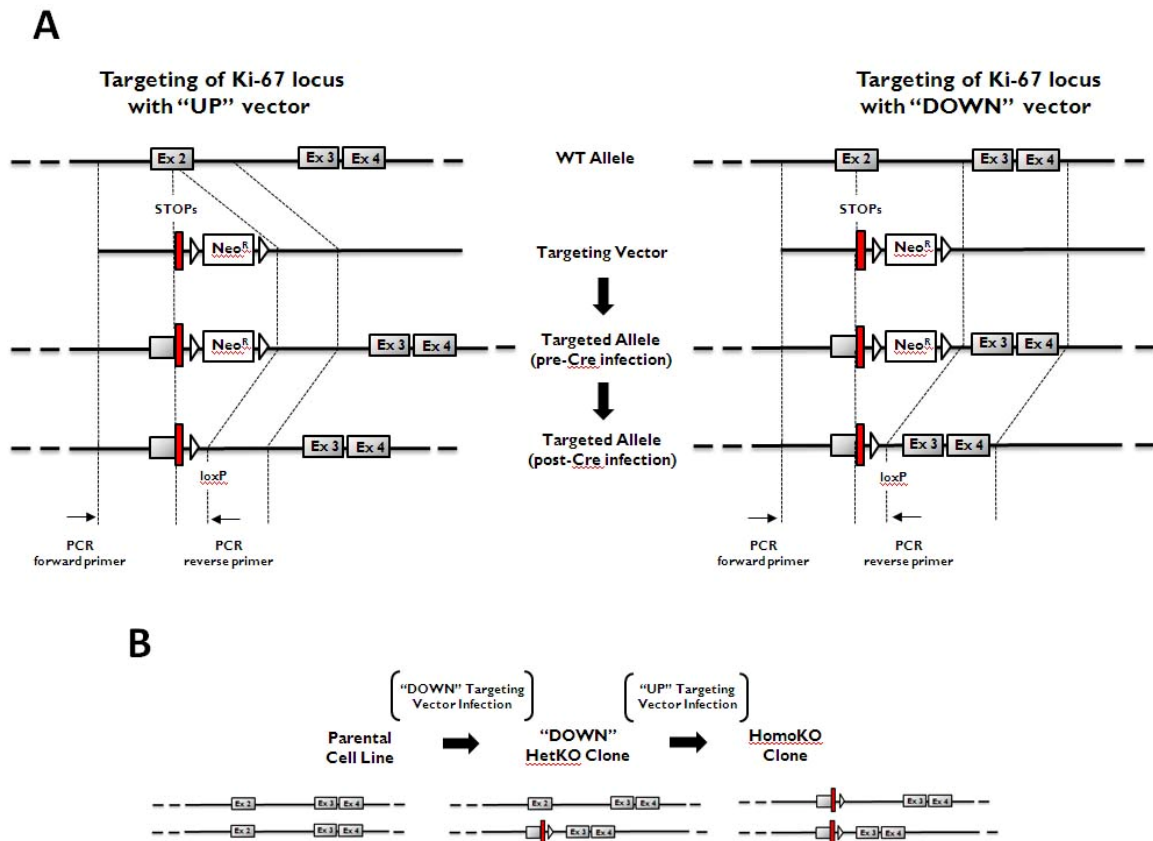
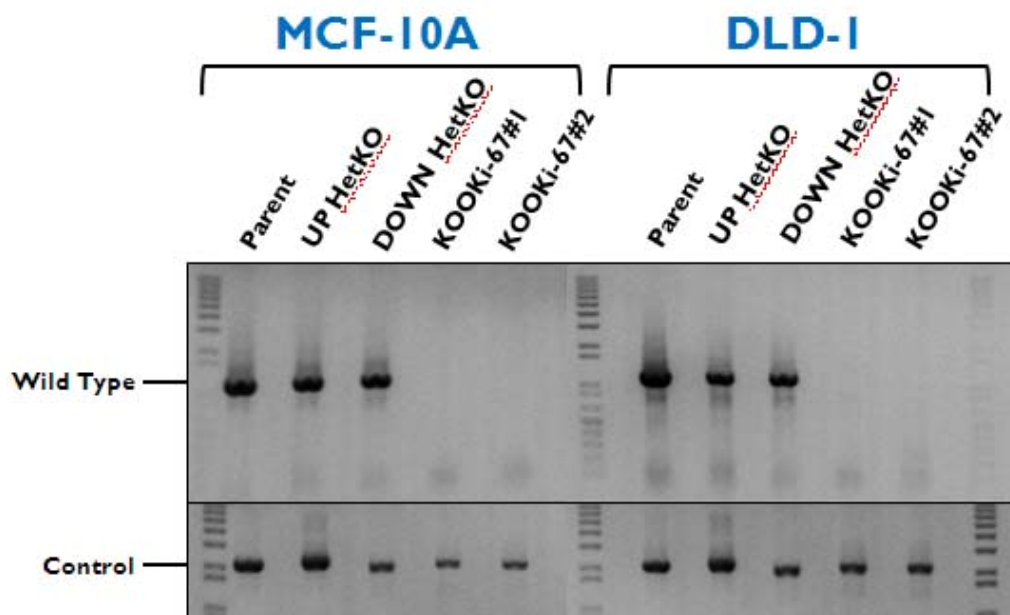
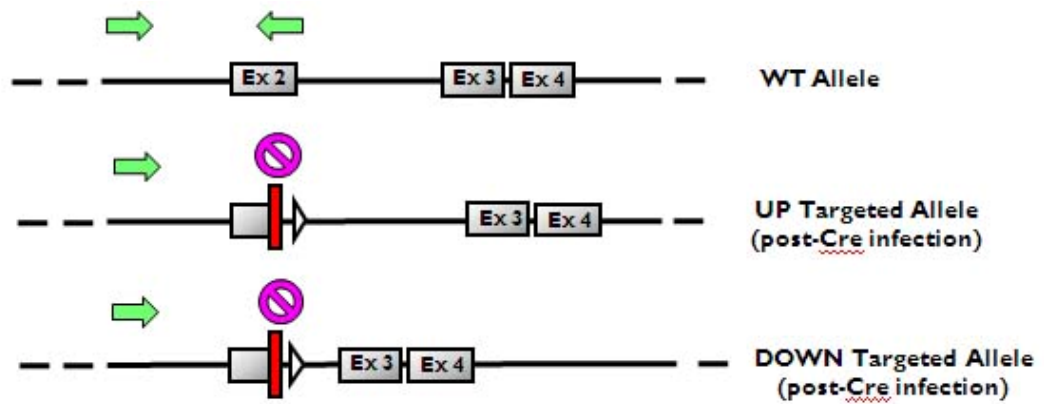


Figure 1: Schematic for targeted knockout of *MKI67*.

A scheme for the targeted knockout of the *MKI67* gene. Shows the endogenous *MKI67* gene locus surrounding the first three coding exons, the UP and DOWN targeting vectors containing the premature nonsense mutations (STOP), the two different targeted alleles, and the targeted alleles post-Cre infection and recombination (**A**). To generate the isogenic knockout cell lines, the vectors were transduced into the respective cell lines in sequential order (first the "DOWN" vector, then the "UP" vector) to achieve maximum targeting and screening efficiency (**B**).

A



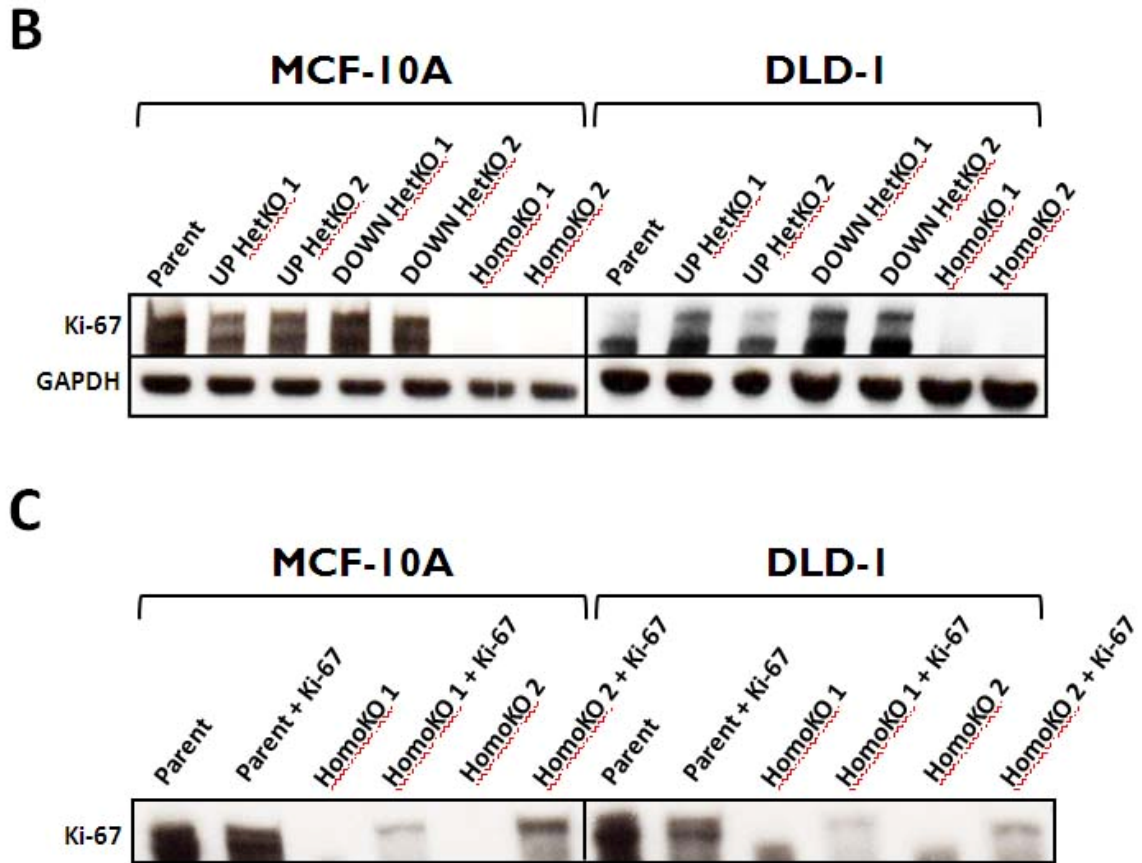
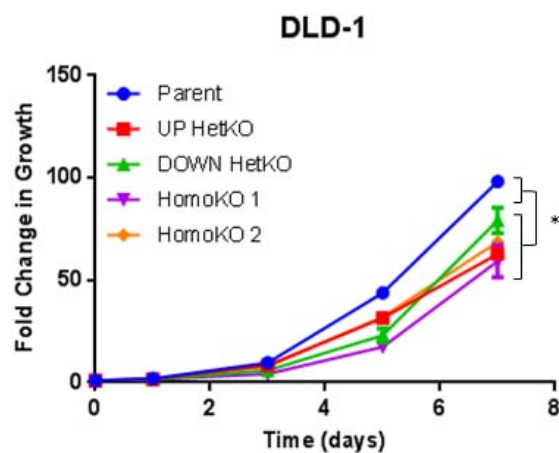


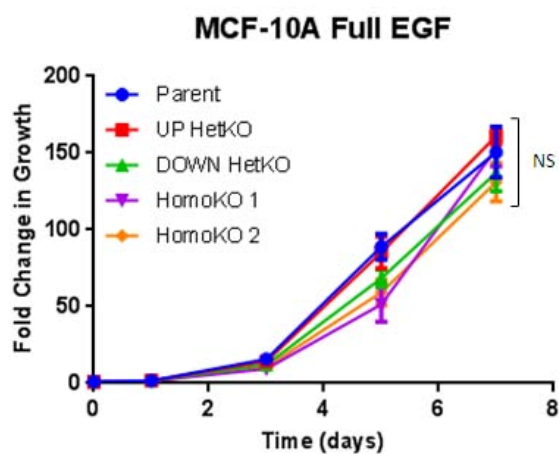
Figure 2: Confirmation of the knockout of Ki-67 in MCF-10A and DLD-1 cell lines.

Upon targeted integration and Cre recombination of the vectors, specific PCRs were performed to confirm the knockout of *MKI67* at each allele by the appropriate construct. The PCR reaction is able to detect a WT allele when present in the cell line since the reverse primer is designed to hybridize to the 13bp deleted by both the UP and DOWN targeting vectors (A). Western blot for Ki-67 protein in the isogenic cell lines for MCF-10A and DLD-1 using GAPDH as an internal control (B – C). Lysates of isogenic cell lines grown under standard conditions (B) or transiently transfected with a Ki-67 overexpression vector to rescue protein expression in the knockouts (C).

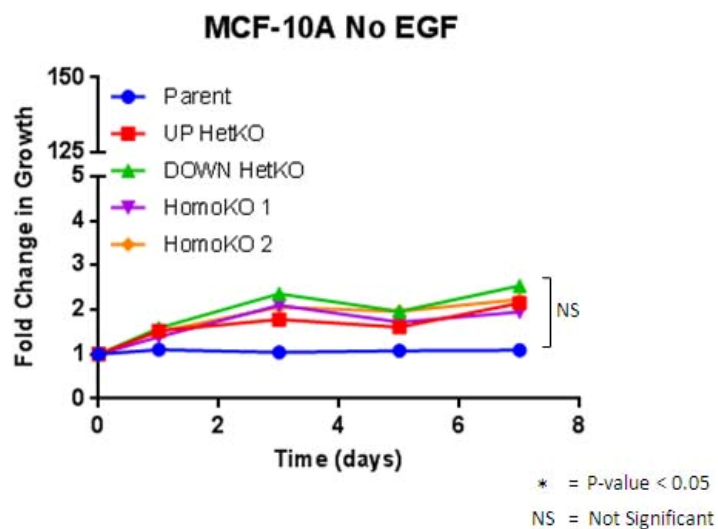
A



B



C



D

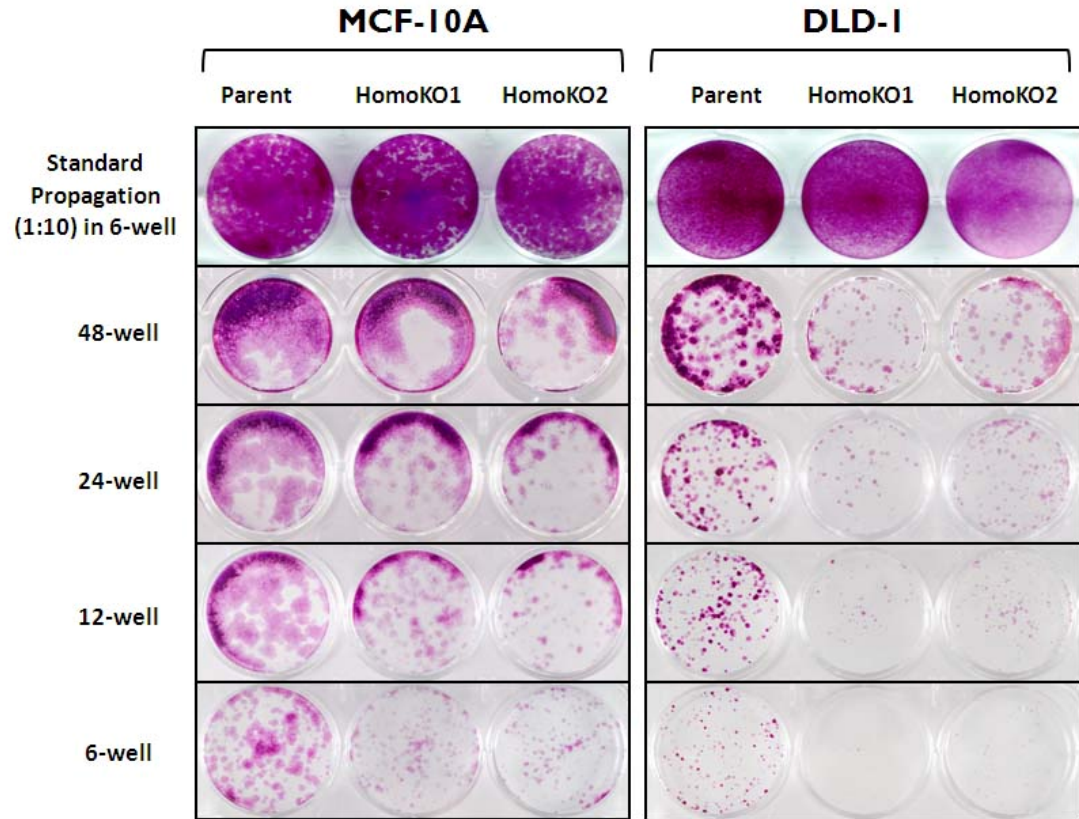
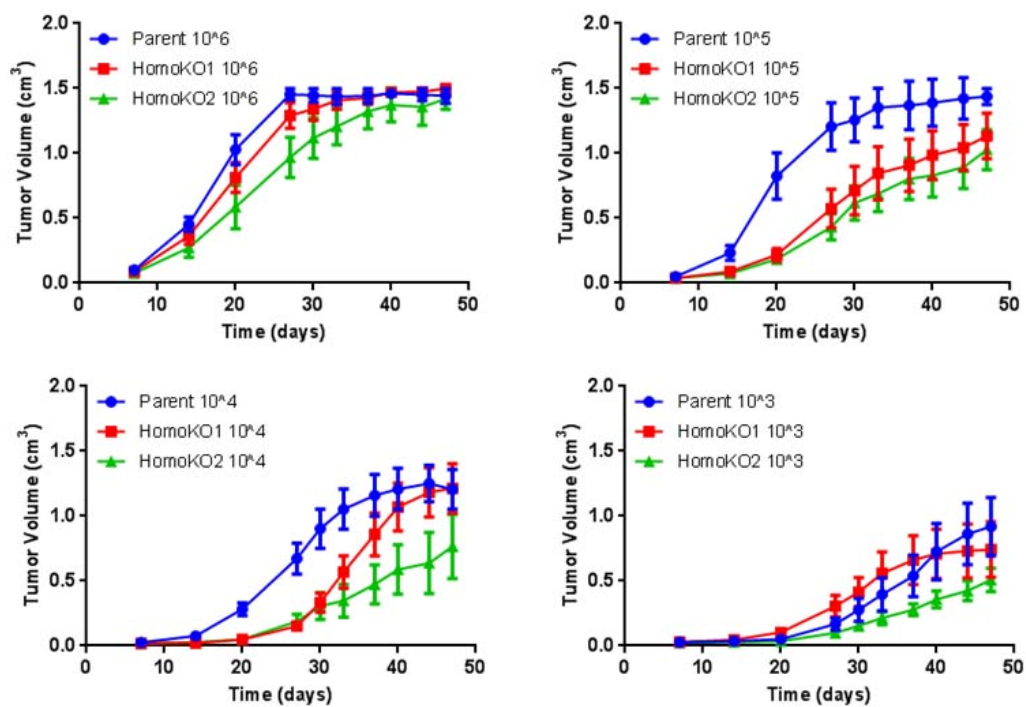


Figure 3: *Reduced proliferative potential of isogenic cell lines in vitro.*

MCF-10A and DLD-1 isogenic cell lines were seeded at 10^3 cells per well in 96-well plates to measure cell growth over a 7-day timecourse via CellTiter-Glo. DLD-1 cells were grown in standard conditions while MCF-10A cells were grown in standard conditions (Full EGF) and in growth arrest conditions (No EGF) (**A – C**). In 48-well, 24-well, 12-well, and 6-well plates, MCF-10A and DLD-1 parental and knockout cell lines were plated in standard conditions, grown for 10 days, and stained with crystal violet (**D**).

A



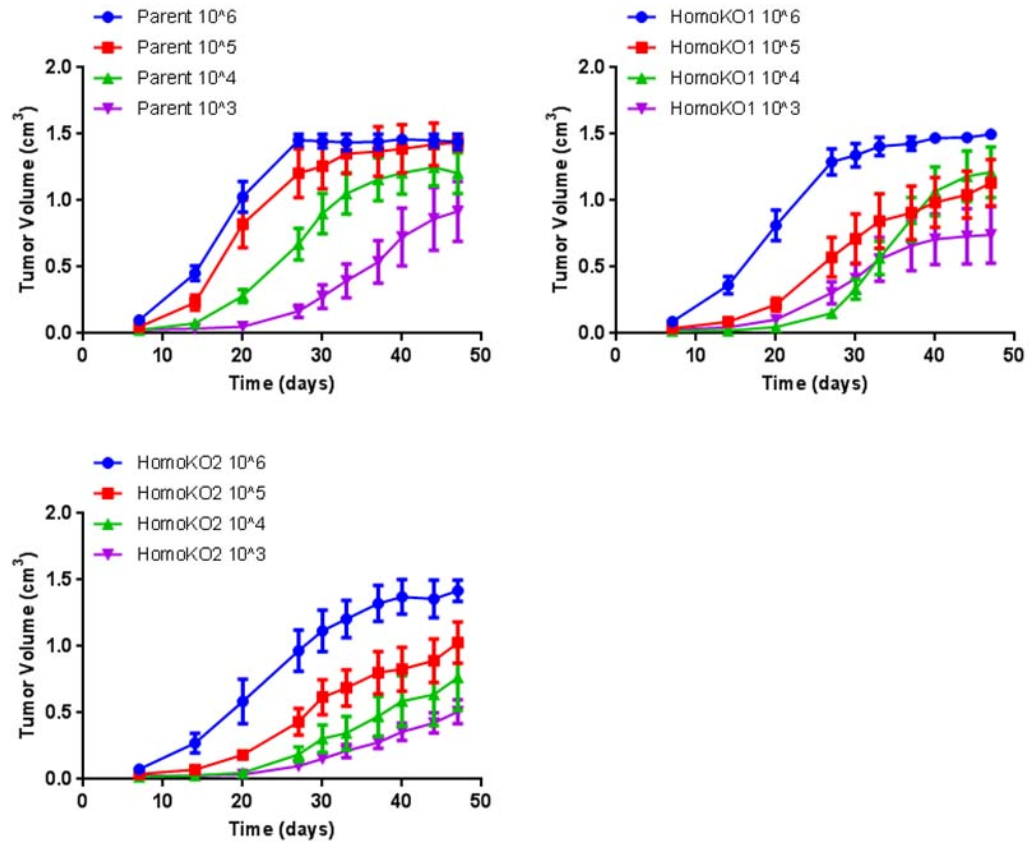
B

Figure 4: *Reduced proliferative and clonogenic potential of Ki-67 knockout cell lines in vivo.*

DLD-1 parent and knockout cell lines were injected subcutaneously into the flank of athymic female nude mice at varying densities. Tumor volume was measured about once per week until the tumor reached a maximal volume of 1.5 cm³. The average tumor volumes were plotted to show the difference between the three clones at the same seeding density (**A**) or the difference between the same clone at the four seeding densities (**B**).

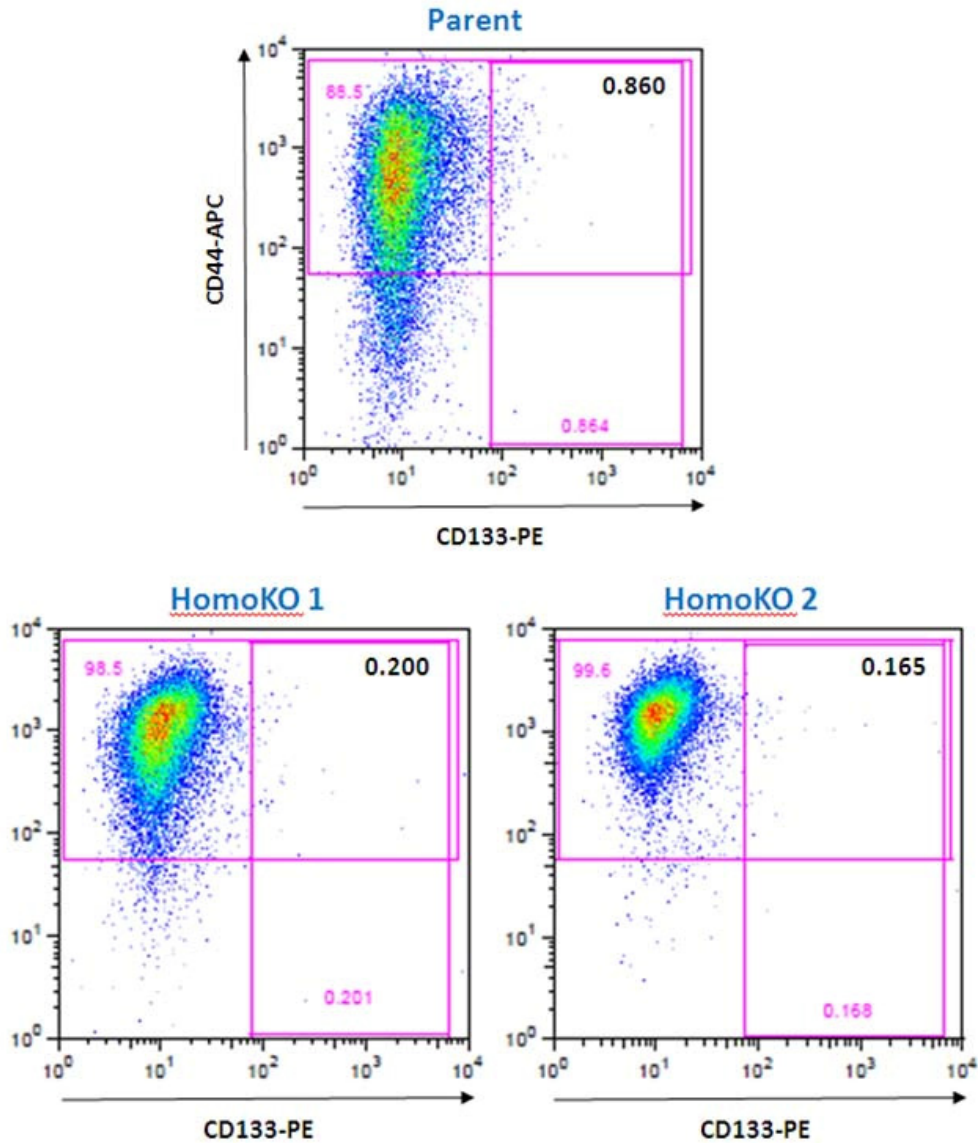


Figure 5: Cancer stem cell population decreased in Ki-67 knockout cell lines.

Flow cytometry to assess the cancer stem cell population. Here are representative data for the CD133⁺CD44⁺ population in DLD-1 parent compared to the isogenic Ki-67 knockout cell lines. Gates for each cell surface marker were created based upon flow cytometry plots of cells treated with the isotype controls.

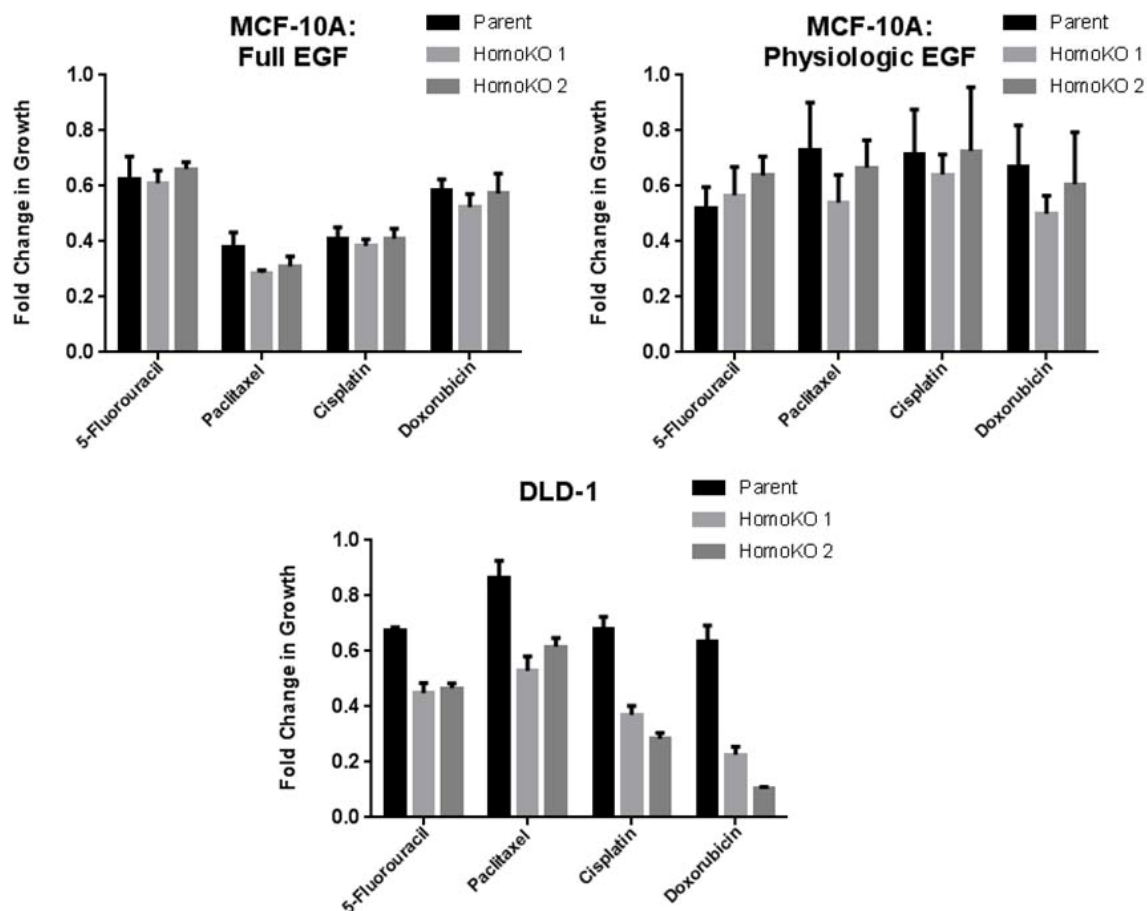


Figure 6: Knockout of Ki-67 in DLD-1 cells sensitizes them to chemotherapy.

Parent and Ki-67 knockout cell lines from MCF-10A and DLD-1 were seeded in standard or indicated growth conditions at 10^3 cells per well in 96-well plates. The next day chemotherapies and vehicle controls were added at doses slightly higher than the IC_{50} for the respective parental cell lines. On day 6, cell viability was assessed with CellTiter-Glo and plotted as fold change in growth relative to the vehicle control and normalized to the measured seeding density of each cell line.

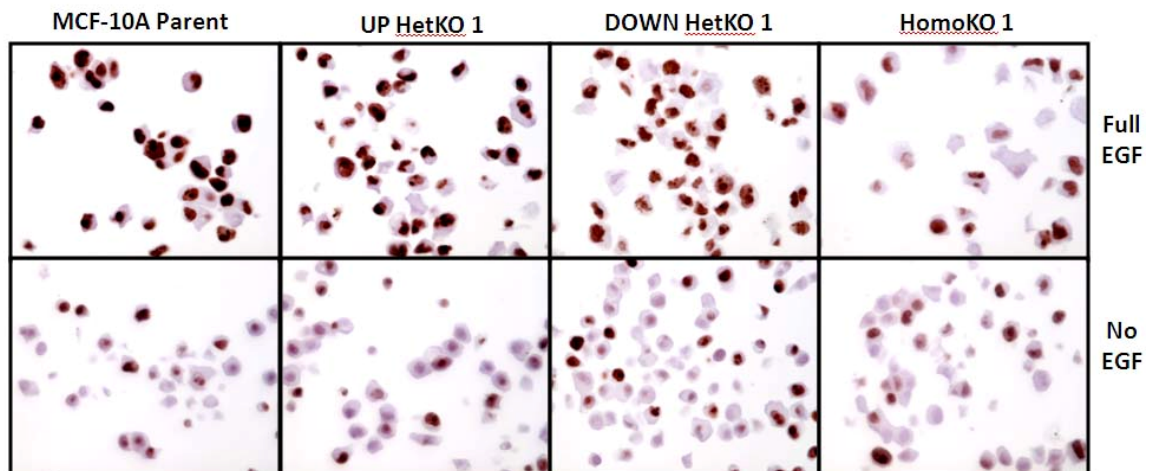


Figure S1: *Confirmation of Ki-67 knockout via IHC of cell tissue blocks.*

Cell tissue blocks were created from MCF-10A isogenic Ki-67 knockout cell lines and stained for Ki-67 protein via IHC. Cells fully growth arrested under No EGF growth conditions displayed non-specific background staining, which is similar to the staining pattern of the knockout cell line grown under standard growth conditions (Full EGF).

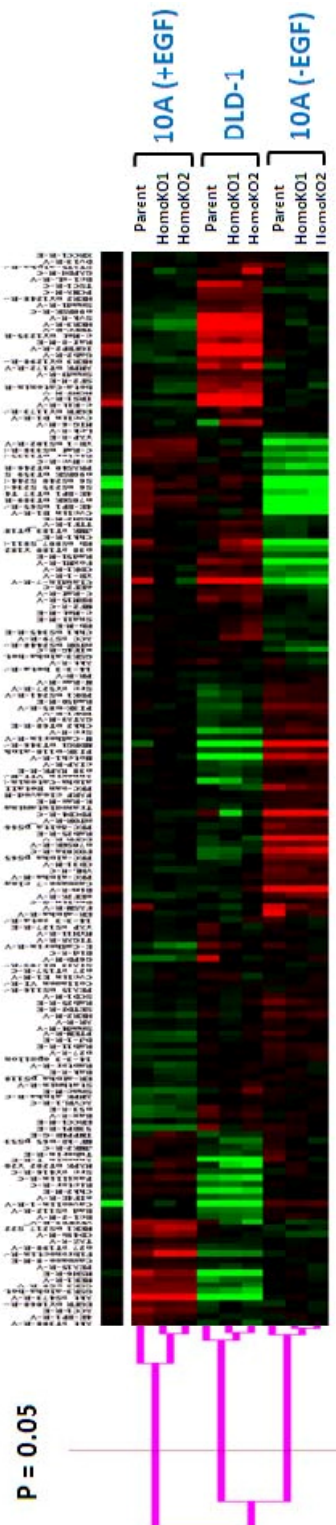


Figure S2: RPPA heatmap for the parent and Ki-67 knockout cell lines.

MCF-10A and DLD-1 parent and Ki-67 knockout cell lines were subjected to RPPA analysis.

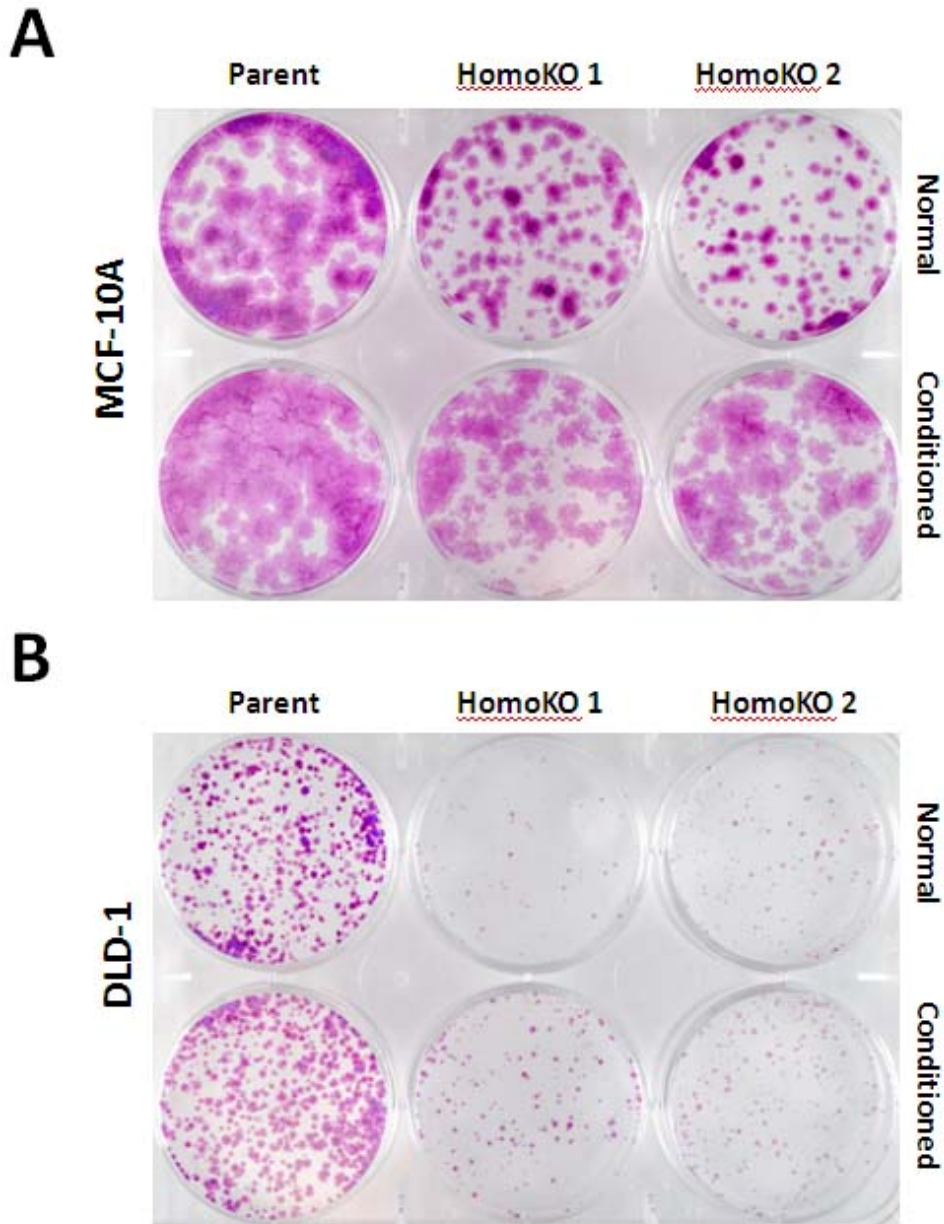
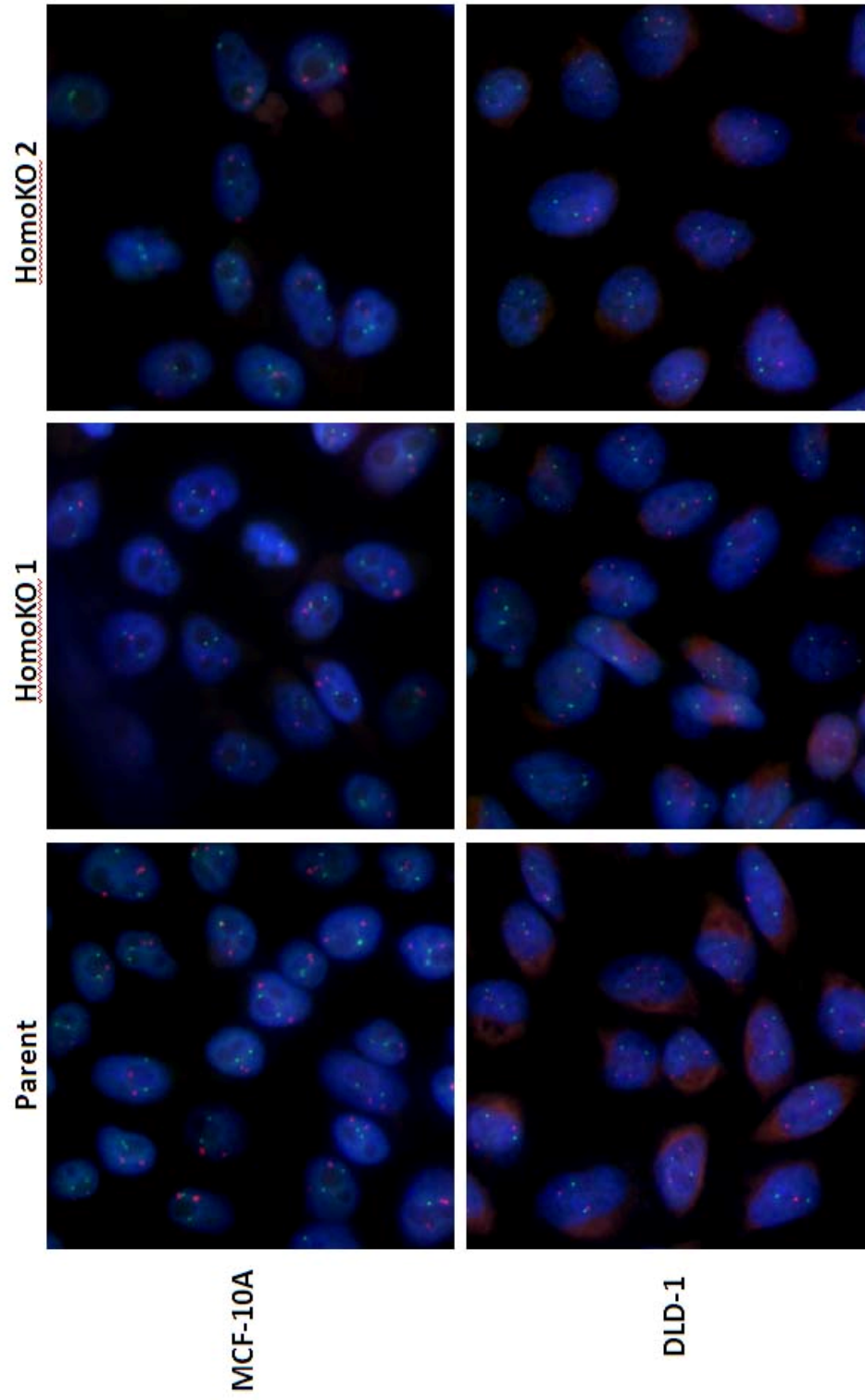


Figure S3: *Conditioned media does not rescue the reduction in growth and/or clonogenic potential of Ki-67 knockout cell lines.*

MCF-10A (A) and DLD-1 (B) parent cell lines were grown to 70 – 80% confluency, and the “conditioned media” was filtered through a 0.2 μm filter. Parent and Ki-67 knockout cell lines were seeded at 10^3 cells per well in 6-well plates and grown over 10 days in either normal or a 1:1 mixture of conditioned:normal growth media.

A



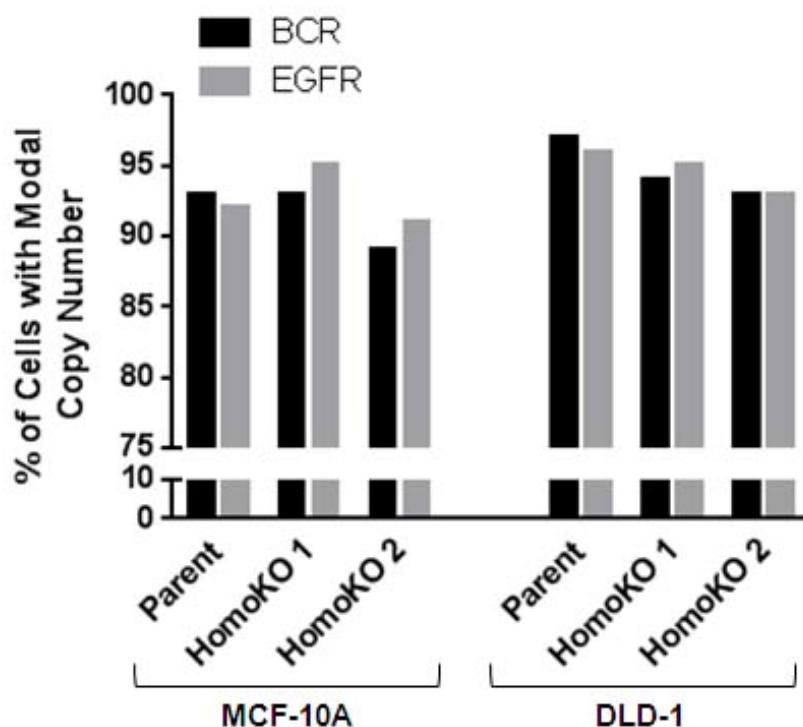
B

Figure S4: *Loss of Ki-67 does not cause chromosomal instability*

FISH was performed on parent and Ki-67 knockout cell lines from MCF-10A and DLD-1 cells to assess for chromosomal instability. Cells were probed for EGFR (red) and BCR (green) loci, with representative experiments displayed. The modal copy number ($N = 2$ for both probes) was determined by counting 200 cells from each cell line, and chromosomal instability assessed as cells deviating from the modal copy number. Chromosomal instability is depicted graphically (A) and quantitatively (B).

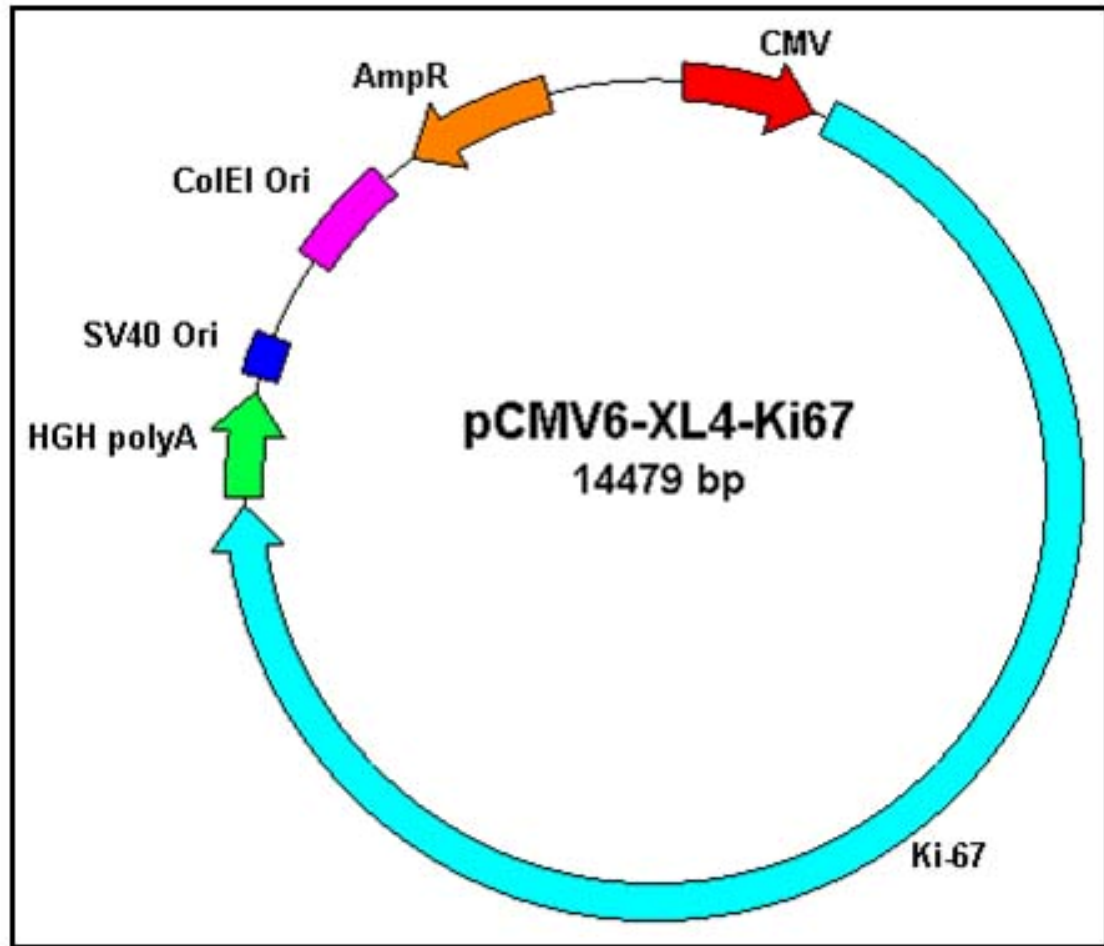


Figure S5: *Diagram of pCMV6-XL4-Ki67 overexpression vector.*

Schematic of the Ki-67 overexpression vector, containing the Ki-67 transcript variant 1 cDNA. Transient transfections to force overexpression of the Ki-67 transgene were done using this vector.

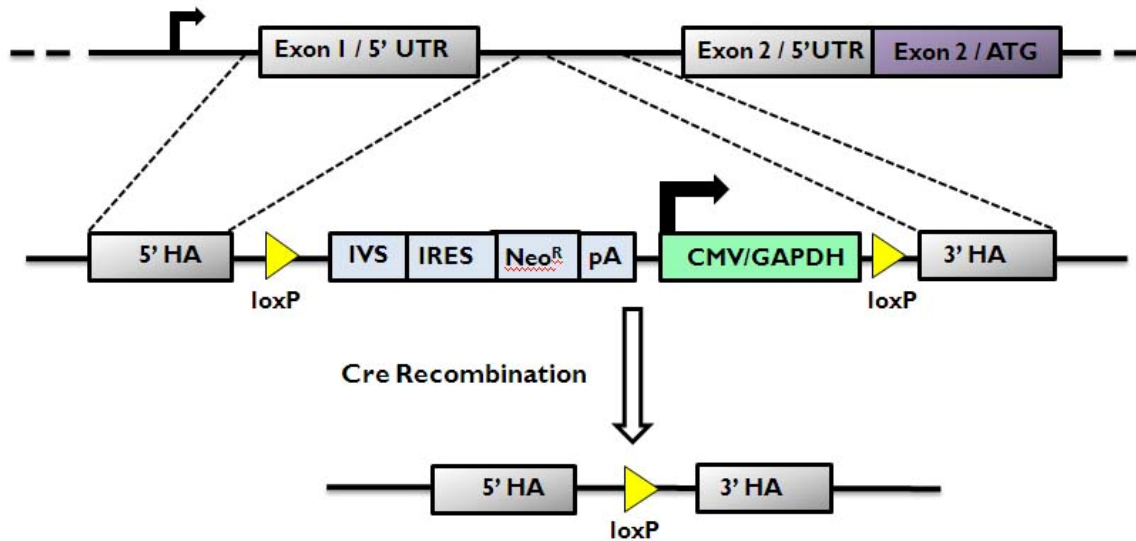


Figure S6: Schematic for knock in of constitutive promoter at the endogenous *MKI67* locus.

This targeting vector contains the usual neomycin selection cassette with that added constitutive CMV or human GAPDH promoter downstream, all of which is flanked by loxP sites. The homology arms flanking the selection cassette and loxP sites targets and integrates within the 5'UTR of the *MKI67* locus so that transcription of the selection cassette is driven by the endogenous promoter. The incorporated constitutive promoter can then drive expression of the full coding region of *MKI67*.

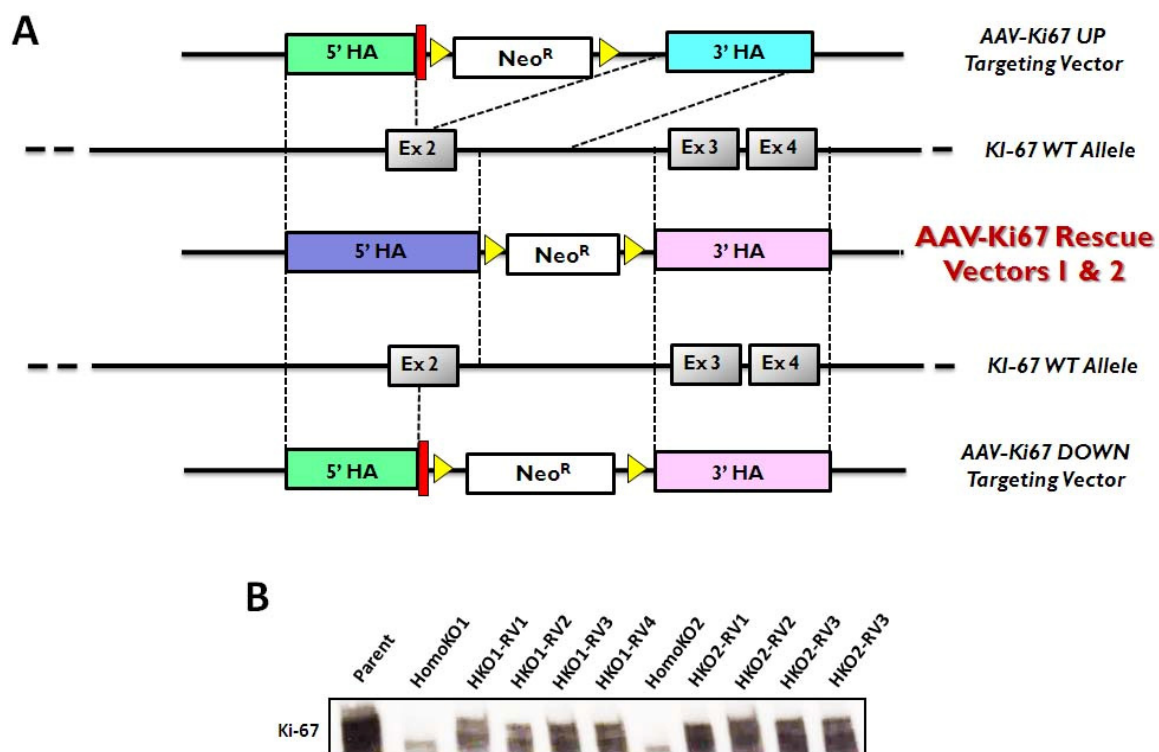


Figure S7: Rescue vector restores Ki-67 expression in knockout cell lines.

Rescue vector re-targets already knocked-out *MKI67* allele, replacing the STOP sequences with the missing coding sequence from exon 2 plus downstream intronic sequence to restore Ki-67 expression. The rescue vector is identical to the DOWN knockout vector with the exception of the extended 5' HA to restore coding and splice sequences (A). Western blot probing for Ki-67 protein in MCF-10A parent, HomoKO clones, and their respective “rescued” clones. Four “rescued” clones were isolated from each of the HomoKO cell lines (B).

Cloning Primers			
Region	Forward	Reverse	Screening
5'HA	GATGCACCCAGGTATTTTC	CCGCTCCTTTGATAGTAACC	GTGTCCCAGATGTTTGGTCC
UP 3'HA	CACTTCCCCTGAGCCTCAG	CACCTGATGCCTGAATGATGG	CTGAAGCTGTGGGGTTGG
DOWN 3'HA	GCACAGAAAGTGCTAGCATC	GCACCCAAATCCTTGAATTCC	CTCATGCAGTTTCAAACAC
Rescue Vector 5'HA	GATGCACCCAGGTATTTTC	GGTGCTTCTCCATTCTGTTAC	GTGTCCCAGATGTTTGGTCC
Overexpression 5'HA	CTTCCTATTGGTCCCATG	CGGGGATAGGTGAATTGG	GTGTCCCAGATGTTTGGTC
Overexpression 3'HA	CCCTTCTTAGCTCCTTTC	GTCATTCCCCTGTGGTC	-

Knockout Confirmation Screens		
Region	Forward	Reverse
UP Allele	GGTTACTATCAAAGGAGCGG	CACCTGATGCCTGAATGATGG
DOWN Allele	GGTTACTATCAAAGGAGCGG	GATGCTAGCACTTCTGTGC
Deleted Region	GTGTCCCAGATGTTTGGTCC	GGAAAGTGGGGACCGTCGAC

qPCR Primers		
Gene	Forward	Reverse
MKI67	CGAGACGCCTGGTTACTATC	GACGTGCTGGCTCCTGTTC
ACTB	GTCAGAAGGATTCCTATGTG	GCCTGGATAGCAACGTACATG

Table 1: *Cloning, screening, and qRT-PCR primers.*

Primers used to clone and screen the knockout, rescue, and overexpression vectors, and primers used for qRT-PCR analysis.

	Case	Tumor Type	Metastasis Site	Phenotype	Ki-67 (%) Primary	Ki-67 (%) Metastasis
Ki-67 (%) Increase	1	Ductal	Lung	HER2+	15	40
	2	Ductal	Brain	Triple Negative	15	50
	3	Ductal	Brain	Luminal	2	75
	4	Ductal	Lung	Triple Negative	30	50
Ki-67 (%) Unchanged	5	Ductal	Brain	Triple Negative	>30	50
	6	Ductal	Brain	Luminal	15	20
	7	Lobular	GI (Luminal)	Luminal	10	10
	8	Ductal	Lung	Triple Negative	25	25
	9	Ductal	GI (Pancreas)	Luminal	25	25
	10	Ductal	Lung	Triple Negative	25 - 30	25
	11	Ductal	Spine	Luminal	20	20
	12	Ductal	Brain	Luminal	20	20
	13	Ductal	Liver	Luminal	10	15
	14	Mammary	Liver	Luminal	5	5
	15	Ductal	Brain	Triple Negative	75	75
	16	Ductal	Brain	Luminal	5	10
	17	Ductal	Brain	Luminal	75	75
Ki-67 (%) Decrease	18	Mammary	Lung	Luminal	10	5 - 10

Table 2: *Change in Ki-67 expression between matched primary and metastatic breast carcinomas.*

Ki-67 staining was performed on matched primary and metastatic breast carcinomas and subsequently scored. Of the 18 samples, 17 displayed either an increased or unchanged expression while 1 showed a borderline decrease.

4 Discussion

Since cancer is a disease of uncontrolled cell growth, it is logical to investigate and attempt to therapeutically target Ki-67, a protein that has been widely accepted as the histological marker for proliferation since its discovery. However, the paucity of knowledge regarding Ki-67 makes it difficult to validate as a drug target. This information gap is primarily due to the difficulty of working with a high molecular weight and highly proteolytically susceptible protein *in vitro*. There is also a surprising lack of functional genomic studies, which may well be the result of conventional wisdom that Ki-67 is essential for the viability of the cell. With our expertise in somatic cell gene targeting, we wanted to overcome the limitations of the few RNAi studies by creating knockout cell lines that completely ablated all functional Ki-67 protein and assess the effects on various cellular processes.

Even though previous functional genomic studies put into doubt the likelihood of creating Ki-67 knockout cell lines, predicting it would result in a lethal phenotype, we

were in fact able to generate both heterozygous (HetKO) and homozygous (HomoKO) Ki-67 knockout somatic cells in MCF-10A and DLD-1 cell lines. Although gene targeting accounts for the questions of specificity and knockdown levels in RNAi studies, the technique is not without its own caveats. Since almost all genes are too large to completely knockout, only select exons are deleted or mutated to incorporate premature nonsense mutations. While this leaves open the possibility for the presence of truncated proteins, great care is normally taken to adequately remove functional domains while nonsense-mediated decay (NMD) typically eliminates the aberrant mRNAs before they can be translated into protein. Through rigorous sequencing, PCR screening, western blot analysis, and IHC, we can definitively confirm that our HomoKO clones lack full length Ki-67. Regardless, there remains the chance, however unlikely, that a truncated form of Ki-67, re-initiated from exon 7 or another downstream exon, escaped our best efforts to eliminate all functional protein.

As mentioned above, we meticulously examined whether we did in fact completely knockout Ki-67 and whether the observed phenotype was not an artifactual result. First, in generating the clones, we calculated about a 9% targeting frequency upon the transduction of the first vector in MCF-10A cells and a 12% targeting frequency in DLD-1 cell lines. If the cells were truly diploid for the *MKI67* gene and only one allele was targeted, we could expect a 50% reduction in targeting frequency upon transduction of the second vector to create the full knockout. In practice, we observed a targeting frequency of 4% in the MCF-10A cells and a 5% targeting frequency in the DLD-1 cells, which gives us considerable confidence that both alleles were properly targeted and knocked out. Both percentages are likely inflated due to our inability to discern between

primarily targeted clones and daughter cells of those primary clones and, therefore, are likely closer to a 2:1 ratio than we observed.

Again, the majority of research conducted on Ki-67 has focused on its temporal and spatial expression patterns. Other areas of interest, such as structure, molecular interactions, and cellular function, have not been explored in any detail. When comparing isogenic knockout cell lines, this lack of information makes it difficult to interrogate very specific areas of interest, such as effects on various signaling networks, leading to more global questions. For instance, we performed RPPA using a panel of over 150 validated antibodies for commonly studied genes. When comparing gene expression changes between the parent and HomoKO cells, we did not observe common trends to both MCF-10A and DLD-1 cell lines. Although the cell line specific expression alterations should be investigated further, it is beyond the scope of this study. Similarly, since Ki-67 has been implicated in higher order chromatin structure and potentially the DNA damage response, we used FISH to assess whether loss of Ki-67 affected chromosome stability, which it definitively did not. While this indicates that Ki-67 likely does not play a role in genomic stability, further research is required to fully understand the role of Ki-67 in chromatin structure and response to DNA damage.

Given the widespread use of Ki-67 as a histological marker for the growing fraction of cells in histopathological samples, the aforementioned RNAi studies attempted to determine if growth arrest or cell death resulted from loss of Ki-67. Likewise, we performed similar growth experiments but came to the opposite conclusion: loss of Ki-67 does not affect the proliferative potential of cells plated for monolayer growth. We did notice a significant difference in growth potential, however, when cells

were plated sparsely for colony formation. Unlike the MCF-10A and DLD-1 isogenic clones grown in monolayer, the HomoKO clones formed colonies at a much slower rate than the parental cell lines. However, the MCF-10A clones still formed the same number of colonies, only at a reduced rate of proliferation. On the other hand, it seems both the proliferative and clonogenic potential of the DLD-1 HomoKO clones was reduced. When we used the DLD-1 isogenic clones to form xenografts of serial dilutions, we observed the same trend. The HomoKO clones formed tumors at a slower rate, but the lower dilutions also seemed to have a reduced clonogenic potential.

We also attempted three different methods to either over express or restore wild type expression of Ki-67. First we tried to stably transfect a Ki-67 transgene into both MCF-10A and DLD-1 HomoKO cells. However, likely due to the large size of the transgene, we were not able to isolate any overexpression clones. Next, through gene targeting, we wanted to insert a constitutive promoter at the *MKI67* locus that would drive expression of the gene. The targeting vector would homologously recombine downstream of the endogenous promoter but upstream of the first coding exon, allowing antibiotic selection to be driven from the *MKI67* promoter while *MKI67* expression was controlled by the integrated CMV or human GAPDH promoter. While we were able to effectively target the locus and isolate targeted clones, the expression level of Ki-67 was not as high as we had expected. In fact, when the targeted clones were propagated under growth arrest conditions, the level of Ki-67 expression was equivalent to cells grown under physiologic EGF concentration (0.2ng/mL). This amount of Ki-67 expression was apparently not enough to overcome growth arrest and drive proliferation. Further to that point, when the targeted clones were grown under standard growth conditions (20ng/mL

EGF), the expression level of Ki-67 was half the amount observed in parental MCF-10 and DLD-1 cells grown under the same conditions. So while the targeted integration of a constitutive promoter at the MKI67 locus did show some low level expression, the clones more closely resembled HetKOs phenotypically rather than overexpression clones. And, lastly, we tried to restore wild type expression of one allele in the HomoKO clones through another round of gene targeting. The rescue vector targeted the same genomic region as the DOWN knockout vector, and the only variation between the two vectors is the 5'HA. While the DOWN knockout vector 5'HA contained the three STOP mutations, the new rescue vector incorporated the deleted region of exon 2 and the adjacent intronic sequence. Although we were successful in restoring wild type Ki-67 protein expression, we were not able to demonstrate a rescued clonogenic phenotype. Some of the isolated clones displayed increased clonogenic potential, approaching the clonogenic capability of parental cells, while others still behaved as the HomoKO cells.

The cancer stem cell (CSC) theory postulates that CSCs are a subset of cells that have the ability to both self-renew and maintain the tumor by giving rise to differentiated, heterogeneous, non-tumorigenic cells that comprise the bulk of the tumor [78, 79]. Only CSCs can maintain tumor growth indefinitely because, although the non-stem cells can actively grow and proliferate, they are destined to die. The initial gold standard for determining the stemness of CSCs was through serial transplantations of small numbers of cells into immunocompromised mice to assess the ability to self-renew and form tumors [86]. Therefore, we questioned whether the reduced clonogenic potential of DLD-1 HomoKO clones could result from the loss of Ki-67 affecting the stem-like nature of the cells. To complement transplantation assays, CSCs have been defined and isolated by

a series of cell surface markers whose expression pattern is unique to each tumor type. For colorectal CSCs, identification remains controversial due to the lack of consensus for molecular markers even though multiple have been proposed. However, the markers most commonly used to identify and isolate colorectal CSCs are CD133 and CD44. Upon subcutaneous injection, CD133⁺CD44⁺ cells always form xenografts [80], and high levels of double-positive cells were a strong indicator for worse disease-free survival and increased risk of recurrence when identified in primary tumors [81]. In another study, CD133⁺CD44⁺ cells were present in each liver metastasis while the primary tumors contained cells with variable expression levels [82]. When we analyzed the parental and HomoKO clones via flow cytometry, there was a noticeable decrease in the percentage of CD133⁺CD44⁺ HomoKO cells. This loss of stemness in the HomoKO clones could explain their reduced clonogenic potential. According to our results, it seems like the loss of Ki-67 somehow affects the CSC population.

It has also been reported that CSCs are more resistant to chemotherapy and/or radiotherapy [83, 87]. While these standard treatments are quite useful at reducing tumor bulk, patients ultimately experience recurrence when deployed as monotherapies. The prevailing theory is that CSCs are resistant to these therapies via drug efflux pumps, alcohol dehydrogenase (ALDH) activity, upregulated anti-apoptotic machinery, and enhanced DNA damage response, thus allowing repopulation of the tumor [88, 89]. Since our analysis of the CSC population in the isogenic DLD-1 Ki-67 knockout clones revealed that loss of Ki-67 is somehow affecting the CSC population, we wanted to confirm this data with a completely separate experiment. Given the known resistance of CSCs to chemotherapy, we hypothesized that perhaps if loss of Ki-67 is affecting the

CSC population then perhaps loss of Ki-67 will affect the sensitivity of the DLD-1 cells to chemotherapy. In this study, when we exposed the DLD-1 isogenic clones to various chemotherapies, the HomoKO clones showed increased sensitivity to the drugs. While this seems to run counter to the clinical model that increased Ki-67 staining predicts for better response to chemotherapy, it has the potential to be a significant finding in the battle against metastatic disease. Combined with the already-reduced tumorigenic potential of Ki-67 knockout cells, treatment with chemotherapy or radiation should hopefully destroy the CSCs along with the bulk of the tumor, significantly inhibiting the ability of the cancer to metastasize.

A study was published last year that examined Ki-67 expression in breast cancer patients pre- and post-neoadjuvant chemotherapy [90]. The authors conclude that it is the final amount of Ki-67 expression in the residual disease that dictates prognoses for the patients. However, in looking at their data, we interpret their results somewhat differently. Since patients who has the highest level of Ki-67 expression to start but were reduced to a “low” level after therapy fared better in disease-free survival compared to patients who actually had “low/intermediate” Ki-67 expression to start and were reduced to “low” levels, we believe that it is the overall change in Ki-67 expression from baseline that should guide prognosis. Given this new understanding, we wondered if patients who have metastatic disease, often considered a “bad” prognosis, would display increased levels of Ki-67 staining in the metastatic tumor relative to their primary site, correlating with the bad prognosis. We were able to stain and score 18 matched primary and metastatic invasive breast carcinomas for Ki-67 expression. In all of the cases, the

metastatic site showed either an increase or no change in Ki-67 staining, confirming that prognostic power of the change in Ki-67 expression post-therapy.

Although we demonstrated that loss of Ki-67 has no effect on cell growth in a bulk population of cells, calling into question its validity as a drug target, the reduced clonogenicity *in vitro* and *in vivo* establishes the possibility that Ki-67 could be a very useful therapeutic target, specifically at the micrometastatic level. While numerous studies have shown that Ki-67 can be used as a prognostic marker in a variety of contexts [57, 60, 61, 63], only a couple examined the Ki-67 status of metastatic specimens compared to the primary tumors [91, 92]. They showed that increased expression of Ki-67 at the metastatic site, relative to the primary tumor, correlated with decreased disease-free survival. Since most cancer deaths result from metastatic disease, validating how therapeutic inhibition of Ki-67 could reduce the rate or likelihood of metastasis has the potential to be a very impactful finding. Ki-67 is still useful as a marker for cell proliferation, and a prognostic and predictive indicator in the clinical setting, but its true utility may be as a therapeutic target in the micrometastatic setting.

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CURRICULUM VITAE

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EDUCATION

- Ph.D. in Cellular and Molecular Medicine** **2014**
The Johns Hopkins School of Medicine, Baltimore, MD
Mentor: Dr. Ben Ho Park, M.D., Ph.D.
Dissertation: Knockout of Ki-67 in Somatic Cancer Cells Reduces Clonogenic Potential and Increases Sensitivity to Anti-Neoplastic Agents
- B.S. in Biochemistry** **2004**
Bates College, Lewiston, ME
Mentor: Dr. Paula Schlax, Ph.D.
Thesis: Regulation of RpoS (σ^S) Translation by Synthetic ncRNAs

ACADEMIC ACHIEVEMENTS & AWARDS

- Polard Scholar, Johns Hopkins CMM Graduate Program **2010**
Graduated Cum Laude from Bates College **2004**
American Cancer Society Fuller Fellowship **2003**
Bates College Dean's List **2000 - 2004**

RESEARCH EXPERIENCE

- Ph.D. Candidate** **2008 – 2014**
The Sydney Kimmel Comprehensive Cancer Center, Baltimore, MD
Utilized viral-mediated targeting vectors to generate isogenic MKI67 knockout somatic cell lines to elucidate its role in normal or cancer cell growth and proliferation.
- Scientific Associate I/II** **2004 – 2008**
Novartis Institutes for Biomedical Research, Cambridge, MA
Developed high-throughput, biochemical assay to investigate the molecular mechanism of action of small molecule inhibitors for an Early Drug Discovery team. Later contributed to Target ID and Validation by elucidating the role of potential gene targets in molecular pathways through genetic manipulation.
- Senior Thesis** **2003 – 2004**
Bates College, Lewiston, ME
Investigated the regulatory effects of small, non-coding RNAs (ncRNAs) on RpoS (σ^S) translation.
- Alvan T., Viola D. Fuller Junior Research Fellowship** **2003**
Boston University School of Medicine, Boston, MA
Investigated the phosphorylative regulation and transactivational properties of tumor-suppressor gene p53 by an upstream kinase.

Center Immunogenetics & Transplantation, Brigham & Women's Hospital, Boston, MA
Focused on the exploration of cardiac gene expression using Real Time Quantitative PCR following vascularized murine cardiac transplants.

PUBLICATIONS

Wang GM, Wong HY, Konishi H, Blair BG, Abukhdeir AM, Gustin JP, Rosen DM, Denmeade SR, Rasheed Z, Matsui W, Garay JP, Mohseni M, Higgins MJ, **Cidado J**, Jelovac D, Croessmann S, Cochran RL, Karnan S, Konishi Y, Ota A, Hosokawa Y, Argani P, Lauring J, Park BH (2013). "Single copies of mutant KRAS and mutant PIK3CA cooperate in immortalized human epithelial cells to induce tumor formation." Cancer Res. 73(11): 3248-61.

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